Characterisation of deletions of the ZFHX1B region and genotype-phenotype analysis in Mowat-Wilson syndrome

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In 1998, Mowat et al delineated a syndrome with Hirschsprung disease (HSCR) or severe constipation, microcephaly, mental retardation, and a distinctive facial appearance. Because two of the patients had a cytogenetically visible deletion of 2q22-q23, and all patients were sporadic cases, a contiguous gene syndrome or a dominant single gene disorder involving this locus were suggested. Two similar patients with cytogenetically balanced translocation (t;2;13)(q22;q22) and (t;2;11)(q22.2;q22), respectively, allowed Wakamatsu et al and Cacheux et al to narrow down the critical interval to 5 Mb and to one single gene respectively, which led both groups independently to the detection of intragenic mutations in the gene coding for Smad interacting protein-1 (formerly SIP1, now called zinc finger homeobox 1B (ZFHX1B)) in patients with so called “syndromic HSCR”. However, because HSCR is not an obligatory symptom and patients with and without HSCR can be recognised by other features, especially their distinct facial gestalt, we suggested that “Mowat-Wilson syndrome” (MWS) is a more appropriate name.

Although the developmental ZFHX1B expression pattern fully explains the clinical spectrum observed in patients with Mowat-Wilson syndrome by haploinsufficiency of this gene alone, Wakamatsu et al initially stated that their deletion patient would have a more severe phenotype and therefore would have a contiguous gene syndrome. Amiel et al reported that the phenotype was similar in patients with “syndromic HSCR” caused by mutations and cytogenetically non-visible large scale deletions of the ZFHX1B locus, respectively, but the deletion sizes were not delineated. We therefore analysed deletion size and genotype-phenotype correlation in four new patients with cryptic deletions of the ZFHX1B locus.

MATERIALS AND METHODS

Patients
The diagnosis of Mowat-Wilson syndrome was made in patients 3 and 4 (fig 1C) because of HSCR and associated features and in patients 1 and 2 because of mental retardation associated with the distinct facial gestalt (fig 1A, B) in the absence of HSCR. Clinical details are provided in table 1; patient 2 will be described in more detail elsewhere.

METHODS

Conventional chromosome analysis was performed from cultivated peripheral blood cells after GTG and CBG banding at a 550-850 band level according to standard protocols. FISH analysis was performed with directly labelled BAC probes on metaphase spreads as described previously.

RESULTS

Results of FISH and marker analysis are shown in fig 2. In patient 3 the distal border of the deletion was determined with polymorphic markers, which showed a distal breakpoint between markers D2S2275 (150.3 Mb) and D2S2299 (152.2 Mb), and a paternal origin of the deletion. Deletions were of differing sizes, approximately 300 kb in patient 4, 700 kb in patient 2, 5 Mb in patient 1, and 11 Mb in patient 3. The mothers of patients 2 and 4 and both parents of patient 3 were available for FISH analysis, which showed normal results. The phenotype observed in patient 3, with the largest deletion, showed early seizures, hypoplastic big toes, and premature death at the age of 4 months as additional features (table 1).

DISCUSSION

Our results indicate that deletion sizes and breakpoints in Mowat-Wilson syndrome patients vary widely, ruling out a true microdeletion syndrome with recurring breakpoints mediated by low copy repeat regions. There was generally no obvious correlation between the phenotype and the size of the deletion and the phenotypic spectrum was similar to that observed in patients with truncating mutations within ZFHX1B (table 1). The only remarkable difference was noticed...
in patient 3 with the 11 Mb deletion, who presented with seizures much earlier, had marked hypoplasia of the big toes, and who died in early infancy. Thus, genes within the close vicinity of the \textit{ZFXH1B} gene seem not to be subject to gross haploinsufficiency. Parental origin was only determined in one of the present and three published patients,\textsuperscript{38} and was of paternal origin in all cases investigated. As all investigated patients had HSCR and congenital heart defects, it is not possible to draw any conclusion about these symptoms, but agenesis of the corpus callosum was present and absent in two patients each, and thus shows no correlation with parental origin of the deletion. Similarly, the early onset of seizures in patient 3 is also not attributable to the parental origin of the deletion.

The most frequently observed major malformation in Mowat-Wilson syndrome is HSCR, which occurred in 21 of 30 (70\%) patients reported so far (table 1). As has been described for patients with \textit{ZFHX1B} truncating mutations, two of our patients with deletions of approximately 700 kb and 5 Mb, respectively, did not have HSCR, while the two with the smallest and largest deletion (300 kb and 11 Mb deletions, respectively) did have it. Thus, our results suggest that the manifestation of HSCR is not influenced by deletion size. As \textit{ZFHX1B} knockout mice also do not exhibit HSCR,\textsuperscript{12} a non-allelic modifier might contribute to the manifestation of HSCR. The high rate of HSCR in humans is probably the result of recognition bias, as in our cohort (four patients reported earlier\textsuperscript{6} and the present four patients) HSCR occurs in only 50\%. Less frequent malformations include various congenital heart defects (for example, septal defects, pulmonary stenosis, or atresia), agenesis of the corpus callosum, urogenital anomalies, talipes, and strabismus.

Similarly, there is no difference in degree of mental retardation, facial appearance, and growth parameters. Regardless of the underlying defect, which may be a truncating mutation in \textit{ZFHX1B} or a large scale deletion, psychomotor retardation is severe with a mean walking age of 4-5 years and speech starting at the age of 5-6 years, being restricted to single words. Personality is generally happy and affectionate. Although shortness of stature and low weight are characteristic in school age children, birth measurements are usually normal or even in the upper normal range. Only microcephaly was already evident at birth in eight out of 19 patients with reported measurements (table 1), and it has a tendency to occur before the decline of body length in our patients. Therefore, our findings do not support the initial statement by Wakamatsu \textit{et al}\textsuperscript{3} about the more severe phenotype in their deletion patient. Nevertheless, severe cerebral atrophy remains remarkable in this patient, but might be related to the other translocation breakpoint on chromosome 13.

A seizure disorder with varying age of onset is a very common feature which is found in 82\% of all 34 patients (table 1). Severe neonatal seizures, however, have been reported only in our patient 3 and a patient with a cytogenetically visible deletion.\textsuperscript{2} Thus a gene(s) responsible for early seizures with a lethal course and hypoplastic big toes might be located between BAC RP11-207O14 at 145.3 Mb and marker D2S2299 at 152.2 Mb where at least one gene related to epilepsy, \textit{CACNB4} (OMIM 601949), is known to be located. However, detailed analysis in further patients is required for confirmation of this putative association.

The characteristic facial appearance was evident in all patients with deletion or truncating mutations and allows the distinction between Mowat-Wilson syndrome and other types of “syndromic HSCR” such as Goldberg-Shprintzen syndrome. The facial features are probably diagnostic in the neonatal period in the presence of HSCR, but the sunken eyes, broad, flared eyebrows, pointed nasal tip, short philtrum, and upturned ear lobes become more obvious in early childhood. Of 12 patients with the distinct facial gestalt of Mowat-Wilson syndrome,\textsuperscript{6} three had the 300 kb deletion and were all of paternal origin. Genetic analysis of parental origin was not available for patient 3. As all investigated patients had HSCR and congenital heart defects, it is not possible to draw any conclusion about these symptoms, but agenesis of the corpus callosum was present and absent in two patients each, and thus shows no correlation with parental origin of the deletion. Similarly, the early onset of seizures in patient 3 is also not attributable to the parental origin of the deletion.
Table 1 Phenotype of previously published patients with Mowat-Wilson syndrome and mutation, deletion, or translocation breakpoint in the ZFHX1B gene and of the present patients

<table>
<thead>
<tr>
<th>MWS patients with mutations (n=22)</th>
<th>13 - 681 41 5 BP in ZFHX1B IVS2 41 5 Del2 Del1 Del351 6 Del 8 Pat 1 Pat 2 Pat 3 Pat 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>16 M, 6 F</td>
</tr>
<tr>
<td>Sporadic occurrence</td>
<td>+ + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Birth length (centile)</td>
<td>5 × &lt;90th 50th 10th 50th 4 × &gt;50th</td>
</tr>
<tr>
<td>Birth weight (centile)</td>
<td>4 × &lt;90th 90th 50th 90th 9 × 50th</td>
</tr>
<tr>
<td>OFC at birth (centile)</td>
<td>4 × &gt;25th 50th &lt;3rd 25th &lt;3rd 8 × 3rd</td>
</tr>
<tr>
<td>Age at last investigation</td>
<td>13 mth - 23 y 6 y 2 y 6 mth 3 y 6 y</td>
</tr>
<tr>
<td>Length (centile)</td>
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<td>Weight (centile)</td>
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<tr>
<td>OFC (centile)</td>
<td>3 × 10th-50th &lt;3rd &lt;&lt; &lt;3rd 3rd &lt;&lt; &lt;3rd</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>18 + + + + + + + + -</td>
</tr>
<tr>
<td>Severe mental retardation</td>
<td>22 + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Walking at age 2-8 y</td>
<td>6 y - - - - - - - - - - - - - - - - - -</td>
</tr>
</tbody>
</table>
| Personality                      | Predominantly happy Happy Happy Happy |}

BP: breakpoint; IVS2: intron 2; ZFHX1B: zinc-finger homeobox protein 1B; OFC: occipitofrontal circumference; MWS: Mowat-Wilson syndrome; HSCR: Hirschsprung disease; short segment (SS); long segment (LS); VUR: vesicoureteric reflux; Age of onset of seizures is given.
syndrome analysed in our laboratory so far (data not shown), eight had truncating mutations and four had large scale deletions, thus giving a ZFHX1B defect in 100% of patients and a deletion rate of 33%. However, it is possible that less severe malformations owing to a non-truncating 3 bp in frame deletions, thus giving a syndrome that is becoming increasingly recognised by clinical geneticists with Mowat-Wilson syndrome now that the clinical features and in time it will be possible to elucidate the true clinical spectrum.

ACKNOWLEDGEMENTS
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REFERENCES
Molecular study of three cases of odontohypophosphatasia resulting from heterozygosity for mutations in the tissue non-specific alkaline phosphatase gene


Hypophosphatasia is an inherited disorder characterised by defective bone and tooth mineralisation and deficiency of serum and bone alkaline phosphatase activity. The bone symptoms are highly variable in their clinical expression and range from stillbirths without mineralised bone to pathological fractures developing only late in adulthood. Odontohypophosphatasia is characterised by premature exfoliation of fully rooted primary teeth and/or severe dental caries, often not associated with abnormalities of the skeletal system. The anterior deciduous teeth are more likely to be affected and the most frequently lost are the incisors. Dental x-rays show reduced alveolar bone and enlarged pulp chambers and root canals. Although the only clinical feature is dental disease, biochemical findings are generally indistinguishable from those in patients with mild forms of hypophosphatasia (adult and childhood). While perinatal hypophosphatasia and infantile hypophosphatasia are transmitted as an autosomal recessive trait, both autosomal recessive and autosomal dominant transmission may be found in childhood, adult, and odontohypophosphatasia. The distinction between recessive and dominant transmission may be difficult to determine conclusively by using familial analysis because expression of the disease is very variable, with parents of even severely affected children showing no or extremely mild symptoms of the disease.

The tissue non-specific alkaline phosphatase (TNSALP) is a phosphomonoesterase anchored at its carboxyl terminus to the plasma membrane by a phosphatidylinositol-glycan moiety. The enzyme cleaves extracellular substrates pyridoxal-5’-phosphate (PLP), phosphoethanolamine (PEA), and inorganic pyrophosphates (PPI). Its exact function in bone and dental mineralisation is still unclear but probably involves hydrolysis of PPI and perhaps mammalian specific activities such as collagen and calcium binding.

The TNSALP gene is localised on chromosome 1p36.1 and consists of 12 exons distributed over 50 kb. More than 127 distinct mutations have been described in the TNSALP gene, in a relatively small number of North American, Japanese, and European patients, indicating a very strong allelic heterogeneity in the disease. Most of them (82%) were missense mutations. This variety of mutations results in variable clinical expression even among the severe or moderate types. We report here the study of TNSALP gene mutations in three patients affected by odontohypophosphatasia and provide evidence that heterozygosity may produce clinical signs and symptoms that appear to be very variable in expression.

MATERIAL AND METHODS

Patients

Patient 1

The proband was a 9 year old boy affected by Down syndrome and odontohypophosphatasia. Loss of seven deciduous teeth,
mostly incisors, began at the age of 2 years. Serum alkaline phosphatase was low (80 U/l, normal range >100). X-rays showed normal growth plate development and normal long bones without evidence of fractures or rickets. The 39 year old mother of the proband had lost her permanent teeth. Her serum alkaline phosphatase level was low (18 U/l, normal range 30-120). The father did not show any symptoms and had a normal level of serum alkaline phosphatase (93 U/l, normal range 30-120).

Patient 2
The proband was a 2 year old male who has lost three teeth and was referred to the genetics department by his dentist. Serum alkaline phosphatase was low (64 U/l, normal range 100-320) and urinary phosphoethanolamine was high (583 µmol/g creatinine, normal range <350). The parents deny any problem with multiple fractures, bowing of legs, or loss of teeth but the 38 year old mother reported being affected by an unusual number of dental cavities and having had numerous treatments of dental root canals. Her serum ALP was low (29 U/l, normal range 47-137) while serum ALP of the proband's father was normal (66 U/l, 47-137). A second child was born in July 2002. At nearly 7 months of age, this baby boy has not shown any symptoms of hypophosphatasia to bring him to clinical attention.

Patient 3
The proband was a 2 year old boy diagnosed with hypophosphatasemia. Serum alkaline phosphatase was repeatedly low (84 and 86 U/l, normal range 104-345) and urinary phosphoethanolamine was high (583 µmol/g creatinine, normal range 108-333). He had very slight leg bowing and was just below the 5th centile for height. The mother had a low ALP level (66 U/l, normal range 47-137) while serum ALP of the proband’s father was normal (66 U/l, 47-137). A second child was born in July 2002. At nearly 7 months of age, this baby boy has not shown any symptoms of hypophosphatasia to bring him to clinical attention.

METHODS
Primer sequences of the 12 ALPL gene exons have been previously reported and allowed analysis of the whole coding sequence, including intron-exon borders and untranslated exons. PCR reactions were performed and analysed as previously described. Site directed mutagenesis of the mutation P91L was performed with the Quikchange Site Directed Mutagenesis kit (Stratagene). Mutated and wild type plasmids were transiently transfected in COS-1 cells using the Lipofectamine PLUS reagent (Life Technologies) according to a methodology described previously. The mutations were put into a 3D model of the TNSALP molecule by using the molecular visualisation program RasMol (R. Sayle, Glaxo Research and Development, Greenford).

RESULTS
Pedigree data and mutation analysis results are shown in fig 1. In family 1, the proband’s mother was affected by early loss of teeth, suggesting that the disease could not be put down to the proband’s Down syndrome condition only and that the disease was dominantly inherited. Sequencing of the ALPL gene showed that the patient and her affected mother carried a 323C>T nucleotide substitution resulting in the missense mutation P91L (fig 2). The presence of this mutation in the affected parent and the absence of any other detectable mutation in the patient is consistent with dominant inheritance. The P91L mutation has not previously been reported in hypophosphatasia patients. We therefore introduced it into the expression plasmid pcDNA-3 by site directed mutagenesis and transfected COS-1 cells with the mutant plasmid. We found that the mutation exhibited 0.4% of wild type activity, that is, no or very low residual enzymatic activity, suggesting that this mutation is not a polymorphism and corresponds to a severe allele.
A99T does not allow any significant in vitro residual activity and shows a negative dominant effect. In family 3, the proband did not show any symptoms of hypophosphatasia and was referred to the genetics department because of hypophosphatasaeemia. We found the heterozygous substitution 1240C>A resulting in the missense mutation L397M of maternal origin. Interestingly, the proband’s second cousin and this cousin’s father were affected by odontohypophosphatasia and carried the L397M mutation found in the proband. Exhaustive sequencing of the ALPL gene of the affected cousin did not show evidence of any other mutation. This suggests that in this family, heterozygotes for L397M may be affected by the disease and that its expression was subject to intrafamilial heterogeneity. The L397M mutation was previously reported by Mumm et al., associated with the D277A mutation, in a patient affected by perinatal hypophosphatasia. This suggests that, like P91L and A99T, L397M is a severe allele. We finally concluded that the disease in these three families resulted from heterozygosity for a severe hypophosphatasia allele.

Localisation of the mutated residues in the 3D model of TNSALP based on the placental ALP structure showed that L397M is located in the crown domain, a mammalian specific region observed for the first time in the placental alkaline phosphatase structure and containing a collagen binding loop (fig 3). Alanine 99 is located in an alpha helix running from the active site to the surface of the molecule near the homodimer interface and supporting D92, S93, and A94, three residues of the active site involved in phosphate binding. By disturbing this helix, mutation A99T could therefore affect the active site. Proline 91 is in contact with the active site and there is no doubt that the change of this residue for leucine has an important effect on the catalytic activity. Thus, the study of the 3D model suggests that these mutations alter the function of the enzyme rather than have a structural effect resulting in the degradation of the molecule. This is consistent with the dominant effect of these mutations and the allosteric properties of the enzyme.

DISCUSSION

Considerable variation occurs in the clinical expression of severe forms of hypophosphatasia, owing to the considerable allelic heterogeneity of the ALP gene. Moderate forms of hypophosphatasia, especially odontohypophosphatasia, are not as well documented. Compared to bone forms of hypophosphatasia, only a few mutations responsible for odontohypophosphatasia have been published in this study, but they suggest that similar variation exists in these forms, at both the clinical and genetic levels. In family 2, the patient was found to be heterozygous for the A99T mutation, a mutation also found in a large family with dominant hypophosphatasia. The probands from the previously reported family were a 6 year old girl and her fraternal twin brother, both affected with premature loss of anterior teeth at the age of 3.5, and abnormal urinary PEA and serum PLP values. In addition to premature loss of teeth, the probands were affected by very slight bone symptoms, such as thin cortical bone of the cranium and multiple radiolucent spots in the cranial bones, but no additional skeletal abnormalities. In this article, the authors point out the intrafamilial clinical heterogeneity of the disease in carriers of A99T since the clinical signs were evident in eight carriers of the mutation and absent in subjects without the mutation and in five carriers. This intrafamilial heterogeneity was also observed in family 3 where the carriers of L397M showed variable expression of the disease, ranging from only hypophosphatasaeemia to perinatal disease associated with multiple fractures. However, the absence of clinical symptoms in the proband could be because of his still young age (2 years), although another second cousin was reported to have lost her primary teeth at the
age of 2 (fig 1). Finally, this report confirms that moderate forms of hypophosphatasia are also highly variable in their clinical expression, owing to allelic heterogeneity but also to other factors that remain to be determined, such as other sequence variations in the ALPL gene, a trans effect of other genes, or environmental factors.

Dominant transmission of moderate forms of hypophosphatasia has been documented in a few families. We here report the case of one additional family with dominant odonto-hypophosphatasia (family 1) and two others in which heterozygotes for a TNSALP gene mutation show clinical symptoms, however variable in expression. In our experience, we failed to detect a second mutated allele in 18% of our hypophosphatasia patients, 70% of them being affected by moderate (childhood, adult, or odonto-) hypophosphatasia (E Mornet, unpublished data). In some of these patients, mutations of the ALPL gene may not have been detected because of their location in intronic or regulatory sequences, or because they correspond to large deletions undetectable by the methodology routinely used here. In others, however, the disease may be the result of heterozygosity and no other mutation needed to be sought. Analysis of the transmission of the odonto-hypophosphatasia phenotype, together with serum ALP level and presence or absence of the mutation, may help to distinguish between the two situations.

The mechanism of dominance remains unclear but probably involves interactions between monomers of the dimeric structure that disturb the allosteric properties of TNSALP. We and others have previously reported that some ALPL gene mutations result in a dominant negative effect owing to complete or partial inhibition of the normal monomer by the mutated monomer in the dimeric molecule. Here, we show that residues mutated in these families are localised in the vicinity of functional regions such as the active site and the crown domain, suggesting that they may have a functional role. This is consistent with the expected localisation of mutations resulting in an inhibitory effect.

References

RPGR mutation associated with retinitis pigmentosa, impaired hearing, and sinorespiratory infections

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Retinitis pigmentosa (RP) is a progressive retinal degeneration that affects about 1 in 4000 of the population. Approximately 15–30% of patients with RP have X linked retinitis pigmentosa (XLRP), which is the most severe form of RP consistently manifesting early in life. Night blindness is usually present in early childhood with loss of peripheral visual fields and ultimately central vision, resulting in registered blindness by the end of the third decade. Female carriers display a broad spectrum of fundus appearances ranging from normal to extensive retinal degeneration.

XLRP is genetically heterogeneous with two major loci, RP2 (Xp11.23) and RP3 (Xp21.1). Both disease genes have now been identified (respectively RP2 and RPGR) with RP2 mutations causing disease in approximately 15% of XLRP families, while RPGR mutations are reportedly more common, accounting for up to 75% of XLRP. Two other rare loci for XLRP have also been described on Xp22 and Xq26-27.

Hong et al. described the phenotype and pathology of an RPGR knockout mouse model. They showed the subcellular localisation of RPGR to the photoreceptor connecting cilia, and in the absence of RPGR partial mislocalisation of essential outer segment proteins. These data suggest a putative role for RPGR in the retina, controlling movement of essential proteins from the inner to the outer segment of photoreceptors via the connecting cilia. Several groups have recently identified a retina specific RPGR interacting protein (RPGRIP1). This protein also localises to the photoreceptor connecting cilia and is thought to be a structural component of the ciliary axoneme. Subsequent mutation screening in patients suffering from retinal diseases has identified mutations in RPGRIP1 as a cause of Leber congenital amaurosis.

In this report, we present the phenotype of a family suffering from XLRP associated with hearing loss, sinusitis, and chronic recurrent respiratory tract infections. To identify the causative gene on the X chromosome, we performed haplotype analysis with subsequent mutation screening of candidate genes. The new phenotype described is associated with a mutation in the RPGR gene, and highlights the significance of RPGR protein kinociliary function in non-ocular tissue.

Key points

- We report a novel systemic phenotype associated with XLRP, with patients suffering from hearing loss, sinusitis, and chronic chest infections, suggesting a mutation in a gene involved in ciliary function.
- The phenotype overlaps those described for primary ciliary dyskinesia and Usher syndrome.
- Genetic analysis of this family has identified a frameshift mutation in exon 8 of the RPGR gene.
- A gene in close proximity to RPGR, TCTEL1, was also examined for cSNPs as a potential phenotypic modifier locus; none was detected.
- Our findings show that mutations in the RPGR gene are associated with a complex phenotype broadening the clinical spectrum of disease, and provide supporting evidence for an essential ciliary function for RPGR in the retina and other tissues.
- RPGR and interacting partners involved in kinociliary function in a variety of tissues may therefore represent attractive candidate genes for other diseases, such as primary ciliary dyskinesia or hearing loss.
subjects (fig 1. II.4, III.4, and IV.5) underwent electrophysiological investigation; subject IV.3 was unable to attend because of renal dialysis. Electro-oculographic responses (EOG), full field electroretinography (ERG), and pattern electroretinograms (PERG) were recorded to incorporate the International Society for Clinical Electrophysiology of Vision (ISCEV) standards.

Genotyping
Microsatellite markers on the X chromosome were used to generate haplotypes of affected, carrier, and unaffected members of the family (primers and conditions available at http://www.gdb.org/). Haplotypes were constructed assuming the minimal number of recombination events. PCRs were carried out in 10 µl reactions in the presence of 1 µCi $\alpha^{32}$P-dCTP, 0.5 U Taq polymerase, 200 µmol/l each of dATP, dGTP, dTTP, and 20 µmol/l of dCTP, 50 pmol of each primer, 30 mmol/l Tris-HCl, pH 8.5, 50 mmol/l KCl, 1.5 mmol/l MgCl$_2$, and 0.01% gelatin. Amplification conditions were 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at the primer specific temperature for 15 seconds, and extension at 72°C for 30 seconds. A final extension followed for five minutes at 72°C. Amplified products were mixed with 6 µl of formamide sample buffer and 3 µl aliquots were electrophoresed in 6% denaturing polyacrylamide gels. Electrophoresis was carried out at a constant power of 90 W for between two and five hours depending on fragment size. The gels were then fixed in 10% methanol/10% acetic acid solution for five minutes, dried onto Whatman paper, and analysed by autoradiography.

Mutation screening
The coding sequence and intron/exon boundaries for the RP2, RPGR, and TCTE1L genes were amplified as described previously. 21-25 PCR products were examined by agarose gel electrophoresis before sequencing. The TCTE1L gene was amplified as described by Roux et al. 25 except exon 1 primers were redesigned (TCTE1L1F - TGAAGTGACGCCTGGCGTTG and TCTE1L1R - AGAGGAAGGGCGGGGTGGG) and annealed at 60°C. An aliquot of each amplification product (8 µl) was then purified by the addition of 1 U shrimp alkaline phosphatase (SAP, Amersham Life Science) and 1 U Exonuclease I (United States Biochemical) in SAP buffer, and incubated at 37°C for 30 minutes followed by 80°C for 15 minutes. Five µl of the purified DNA sample was then used for cycle sequencing using Big Dye Terminator cycle sequencing kit following the manufacturer’s instructions. Reactions were then electrophoresed on an ABI 373A automated sequencer.

RESULTS
During the genetic and clinical analyses of families diagnosed with XLRP, a family with additional systemic features was identified. The four generation pedigree structure of this family is shown in fig 1.

Clinical assessment
Ophthalmic phenotype
Affected males manifested night blindness in early childhood, had constricted visual fields by early teens, and were registered as legally blind by 20 years of age. Gross fields to confrontation in affected males IV.3 (aged 25) and IV.5 (aged 16) were less than 10°. Intraretinal bone spicule pigmentation was observed in IV.3 (fig 2). Female subjects II.4 and III.4 were asymptomatic, had normal fields to confrontation, and showed sparse peripheral intraretinal pigmentation (fig 2). Carrier females and affected males were myopic. Visual acuities were as follows for the right and left eyes respectively: II.4, 20/30 and 20/20; III.4, 20/40 and 20/30; IV.3, 20/30 and 20/30; and IV.5, 20/30 and 20/30. Levels of myopia were recorded for carrier females II.4 (5.00 to −1.25 diopters) and III.4 (−7.50 diopters) and III.4 (−10 diopters). Electrophysiological phenotype was ascertained in one affected male patient (IV.5) and two carrier females (II.4 and III.4, fig 3). No recordable ERG could be
detected in the affected male (IV.5) under any stimulus conditions; PERG was also undetectable confirming severe macular involvement. Both carrier females (II.4 and III.4) show delayed 30 Hz flicker ERG with mild delay also present in photopic single flash b wave. Rod specific ERGs do not show definite abnormality, but maximal response a wave is mildly subnormal in subject III.4. PERG is subnormal in both carrier females tested. EOG light rise was abolished in IV.5 and reduced in the carrier females. The ocular phenotype therefore fits the classical description of X linked retinitis pigmentosa, with very severe ERG abnormality in affected males and abnormal ERGs in female heterozygotes in keeping with carrier status.

Systemic phenotype
In this family, however, the classical ocular features of XLRP were associated with additional systemic symptoms in both hemizygous males and heterozygous females.

One of the most striking and obvious additional features was that affected subjects and carriers required hearing aids. Both males and females suffered severe recurrent ear infections from very early childhood continuing into adulthood. All affected males, and carrier female III.4 (aged 44), had progressive hearing loss and required hearing aids. Affected male I.1 (who had died) also had hearing loss and was described as deaf. The exception is carrier female II.4 (aged 68), who had recurrent ear infections, but did not require a hearing aid. The family had not been exposed to harmful environmental influences, such as excessive noise. Predominantly high frequency hearing impairment was recorded for affected male III.8 by pure tone audiometry (PTA, fig 4). Both left and right ear are similarly affected showing hearing loss between 4000 and 8000 Hz. Averaging the thresholds at these two frequencies is the convention to describe the degree of hearing loss predominant at high frequency. Thus, \( \frac{20 + 25 + 40 + 45}{4} = 32.5 \), which indicates that hearing loss in this subject is mild, since the averaged threshold is less than 40 dB hearing loss. The audiogram could be consistent with a diagnosis of sensorineural hearing loss, in view of the hearing loss in the high frequencies. However, a conductive hearing component may also have contributed to this phenotype. Unfortunately, PTA data for other members of the family are not available.

In addition to retinitis pigmentosa and hearing loss, affected males and carrier females also suffered from severe recurrent sinus infections resulting in sinusitis. The three affected males experienced chronic recurrent chest infections starting in early childhood, with episodes of bronchitis, which continued into adulthood. Affected male IV.3 had nasal polyps and renal failure. The patient's affected brother (IV.5) and affected uncle (III.8) did not have renal failure, and carrier females do not seem to suffer from milder renal insufficiency. Although renal failure does not, therefore, segregate with the visual and hearing loss in this family, it remains possible that renal failure in patient IV.3 is part of the spectrum of symptoms for this syndrome.

In summary, the three affected males in this family had classical XLRP associated with hearing loss, sinusitis, and chronic recurrent chest infections, with renal failure in one person. Carrier females (II.4, III.2, and III.4) had a milder systemic phenotype, suffering recurrent ear infections and sinusitis without the chronic chest infections. Obligate carrier

Figure 2 Fundus photos of (A) affected male IV.3, right macula showing retinal thinning and (B) typical intraretinal bone spicule pigmentation in peripheral retina, (C) right macula of carrier female III.4 showing mild retinal thinning, and (D) sparse intraretinal bone spicule pigmentation in the periphery.
III.4 had progressive hearing loss and required a hearing aid. The ocular phenotype of II.4 and III.4 was consistent with carrier status for XLRP. Unaffected members of this family had no symptoms of either the ocular or systemic phenotype described.

The phenotype for this family is therefore XLRP associated with progressive hearing loss, sinusitis, and chronic recurrent chest infections.

Haplotype analysis and mutation screening

Since disease in this family appeared to be X linked through pedigree structure and phenotypic evaluation, we performed haplotype analysis using X chromosome markers to locate the disease gene interval. Haplotype analysis showed disease segregation between the markers DXS1214 and DXS986 spanning the RP2 and RPGR genes. The RP2 gene was screened for mutations by PCR exon amplification followed by direct sequencing. No mutations were identified in this gene. However, a polymorphism was identified in exon 3 (844C>T) which did not segregate with the disease, thus refining the disease interval (fig 1).

Subsequently, RPGR was then analysed in our family. We detected EST matches from olfactory and lung epithelial cells by BLAST and NIX analysis and UniGene database searches (http://www.ncbi.nlm.nih.gov/ and http://www.hgmp.mrc.ac.uk/) suggesting that this ubiquitously expressed gene is present in the tissues of interest. Sequence analysis of RPGR showed a 2 bp deletion in exon 8 (845-846delTG, fig 5). This frameshift mutation at residue 262, which segregates with disease, is predicted to introduce 19 new amino acids and a premature stop codon, resulting in a truncated protein of 280 residues. All exons upstream of exon 8 were also entirely sequenced to exclude the possibility of a second alteration, which may have a combinatorial effect with the exon 8 mutation; no other alterations were detected.
It is likely that the genetic background of subjects within this pedigree contributes to the additional systemic phenotypes observed. Since overlapping symptoms have been observed in numerous XLRP patients and at least one other XLRP pedigree (see Discussion), we hypothesised that a predisposing locus could be closely linked to RPGR on the X chromosome. The TCTE1L gene is approximately 500 kb distal to RPGR and has been shown to be expressed in lung, trachea, kidney, and brain, among other tissues and detects ESTs from olfactory and lung epithelial cells and the organ of Corti. The TCTE1L protein forms part of the cytoplasmic dynein light chain of the microtubule motor complex, and may be involved in tissue specific cargo binding activities since other members of this protein family mediate specific interactions, for example, with rhodopsin. The TCTE1L gene therefore presented an attractive positional and functional modifier locus for the phenotype described. To determine if a linked locus could predispose subjects to susceptibility to the systemic pathology observed, we screened the five exons of the TCTE1L gene for cSNPs and no polymorphic variants were detected in the affected males.

**DISCUSSION**

**Phenotypic overlap with other syndromes**

The systemic phenotype in this family has similarities with those observed in immotile cilia syndrome (ICS1) or primary ciliary dyskinesia (PCD, MIM 242650). PCD is a congenital respiratory disease characterised by impaired mucociliary clearance caused by cilia ultrastructural abnormalities. PCD patients suffer from chronic bronchiectasis and sinusitis, usually associated with male infertility, but do not reportedly suffer from recurrent ear infections or deafness. Approximately half of the patients with PCD also display situs inversus (Kartagener syndrome, MIM 244400). One causative gene for PCD has recently been identified, DNAL1, a dynein intermediate chain gene on chromosome 9p13-21, with mutations in this gene shown to cause axoneme ultrastructural abnormalities in two families. Genetic and phenotypic heterogeneity are features of PCD, with a locus identified on chromosome 5p14-15, and potential linkage to 11 other chromosomal intervals. However, no linkage to the X chromosome or association with RP and deafness has previously been reported.

Usher syndrome is defined by an association of sensorineural deafness with RP with three distinct clinical subtypes (I, II, and III) of variable severity and extensive genetic heterogeneity. Usher syndrome is the most frequent cause of sensorineural deafness accompanied by blindness, although two of the causative genes have also been implicated in isolated deafness or isolated retinitis pigmentosa.

Although hearing loss in our family is associated with retinitis pigmentosa, the mode of inheritance, nature, and onset of hearing loss, and chronic infections leading to sinusitis and bronchitis distinguish the phenotype in this family from Usher syndrome types I, II, and III. Hearing loss in our family appears relatively mild and progressive with a sensorineural component, but the chronic infections suffered by the patients may also contribute to an acquired conductive hearing impairment. Unfortunately, we were not able to record bone conduction thresholds in this family, so we conclude that hearing loss is likely to be mixed, but not proven. It is difficult to say whether the patients have any loss of vestibular function without thorough neuro-otological evaluation, but family members did not report any dizziness/balance problems. The lack of reported symptoms may have been because of the progressive nature of the condition which allowed for vestibular compensation. The presence of an X linked form of Usher-like phenotype has been suggested, but no locus on the X chromosome has been described. In one report, however, retinitis pigmentosa with deafness (described as Usher syndrome) was associated with bronchiectasis and immotile cilia syndrome, and the possibility of an X linked mode of inheritance could not be excluded.

The major sites of pathology in this new phenotype, causing hearing loss and other disabling systemic abnormalities in association with XLRP, suggest structural, degenerative, or developmental kinociliary defects.

**RPGR mutations and ciliary abnormalities**

Several lines of evidence support our findings that mutant RPGR causes XLRP with associated generalised cilia abnormalities. In 1992, Van Dorp et al reported a family who suffered from XLRP with associated susceptibility to respiratory infections in the majority of affected males. The patients suffered from recurrent bronchitis and sinusitis, described as indistinguishable from immotile cilia syndrome, but did not suffer sterility or deafness. In a subsequent publication, a mutation was identified in this family in the RPGR gene, namely a G to T transversion at position +1 of the 5′ donor splice site of intron 5, predicted to result in aberrant splicing. This additional phenotype, reported by Van Dorp et al, overlaps the one described here with the exception of the associated hearing loss, present in our family. Independent studies describing the prevalence of deafness in association with RP identified a group of patients that did not fit into previously described clinical categories (that is, not Usher syndrome). Hearing impairment in three families with XLRP was reported by Rosenberg et al, and a mutation in RPGR had previously been identified in one of these families. This mutation is described as a 6.4 kb deletion which disrupts the...
3' end of RPGR removing the last six exons. Both affected males and carrier females from this family had hearing difficulties.

Other studies have centred around examination of cilia in patients with RP. Several reports examining nasal mucosa and sperm in heterogeneous groups of patients suggest increased incidence of abnormal cilia in XLRP patients. However, the molecular basis for these observations remains undetermined. Although the physiological role of RPGR in retina and other tissues is not fully understood, compelling evidence for the importance of RPGR in ciliary function comes from recent studies of animal models and the identification of RPGR interacting proteins. It is possible, therefore, that other cell-specific ciliary proteins exist in the lung, trachea, inner ear, and nasal passages which bind RPGR, and that this interaction may be compromised in the family described in this report.

**Mutation type and modifiers of phenotype**

The novel mutation we have identified results in partial loss of the RCC1 domain (exon 8, 845-846delTG) and downstream sequence. This protein truncation mutation occurs upstream of many other protein truncation and missense mutations previously reported to cause XLRP. It is unclear, however, why the phenotype of this protein truncation mutation is different from others reported to result in only an ocular phenotype. Perhaps persistent but milder systemic infections in other families remain undetected or may not have been reported, and exposure to infections and subsequent disease manifestation varies widely.

Mutations in the RPGR gene have also been detected in families with X linked cone-rod dystrophy and X linked macular degeneration (as opposed to the rod-cone degeneration observed in classical XLRP), widening the clinical spectrum associated with mutant RPGR and highlighting the fact that other factors modulate the phenotype. The factor(s) underlying the significant variability of the pathogenic expression of RPGR remain to be identified.

The genotype at a particular locus may account for an inter-individual susceptibility that can both increase or decrease the risk to develop the pathology by modulating mechanisms involved in the pathogenesis. We hypothesised that a closely linked gene which segregated with the primary RPGR mutation could be acting as a modifier gene in this family, since association with the symptoms described in this report are more common than previously suspected. TCE1L lies approximately 500 kb distal to RPGR and presents an interesting functional candidate which is expressed in the tissues involved in the systemic disease associated with XLRP. No cSNPs were identified. Predisposing SNPs may lie outside those gene regions tested, and other loci on the autosomes can not be excluded as predisposing factors; however, if autosomal SNPs are involved in disease expression, they are predicted to be common owing to the occurrence of disease in more than one pedigree. It is now essential to collect a cohort of families with these overlapping phenotypes to determine the factors involved in disease expression.

Further evidence for this new syndrome being primarily an RPGR gene disorder comes from colleagues who have identified a family with an almost identical phenotype, XLRP, hearing loss, and recurrent respiratory tract infections. On the basis of our findings, they examined the RPGR gene and found a missense mutation in exon 6 (Iannaccone et al., in preparation). The data show that the families are unrelated and that different mutations in RPGR can result in overlapping phenotypes implicating ciliary dysfunction in a variety of tissues. In addition, Iannaccone et al describe expression of RPGR in human cochlea and bronchial and sinus epithelial lining.

Future studies towards unravelling the function of RPGR in the retina will need to be expanded to include analyses of multiple ciliated epithelial tissues. The identification of RPGR binding partners within these tissues may identify other specific proteins capable of interacting with RPGR. It would be of interest to evaluate the mouse and dog models of RPGR disease with a view to examining structure/function and development of the ciliated epithelium of the respiratory tract, sinuses, and inner ear, for example, in addition to re-evaluating the patients already described as harbouring RPGR mutations as a cause of XLRP.

**SUMMARY**

In conclusion, we describe a new phenotype of typical X linked retinitis pigmentosa associated with hearing loss, chronic respiratory tract infections, and sinusitis caused by a mutation in RPGR. The systemic phenotypes are predictable to be variable, accentuated by repeated infections of the respiratory tract and consequent upon impaired mucociliary clearance (as described for PCD). Phenotypic variation between families may be caused by RPGR mutation type, genetic background, environmental effects, or a combination of these factors. Additional families will need to be investigated for SNPs on the X chromosome in proximity to RPGR to explore fully any phenotypic modification caused by adjacent loci. RPGR and interacting partners involved in kinesin function in a variety of tissues may also represent attractive candidate genes for other phenotypes such as primary ciliary dyskinesia or isolated hearing loss.

**ACKNOWLEDGEMENTS**

The authors would like to thank the family for participating, and The Welcome Trust (AJH) and the British Retinitis Pigmentosa Society for their support.

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Evidence of *RPGRIP1* gene mutations associated with recessive cone-rod dystrophy

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Con-rod dystrophies (CRD) are forms of inherited retinal dystrophy which characteristically lead to early impairment of vision. An initial loss of colour vision (cone mediated functions) and of visual acuity, usually from the first or second decade of life, is followed by night blindness (largely rod mediated) and loss of peripheral visual fields. CRD patients suffer from severe photophobia and show reduced ERG responses. In later life, vision may be reduced to a bare perception of light. CRD is a milder condition compared to Leber congenital amaurosis (LCA) which is the most severe form of all the inherited retinal dystrophies and is diagnosed as bilateral congenital blindness, with a diminished or absent electroretinogram (ERG). Cone-rod dystrophy loci have been mapped to chromosomes 17q, 19q, 18q, 17p13, 1q12, and 8p11. Mutations in the *peripherin/RDS, CRX, RetGC-I* and *RPGRIP1* genes have been shown to cause autosomal dominant CRD. Mutations in the ATP binding cassette transporter rim protein (ABCR) gene have been shown to be associated with autosomal recessive CRD. Mutations in the *CNGA3* gene encoding the α-subunit of the cone photoreceptor CNGα gated channel have also been reported to cause cone photoreceptor disorders.

The *RPGRIP1* protein (retinitis pigmentosa GTPase regulator interacting protein 1, MIM 605446) is encoded by the gene located on chromosome 14q11. It consists of 24 exons and the predicted size of its protein product is 1259 amino acids. It is located on chromosome 14q11. It consists of 24 exons and the predicted size of its protein product is 1259 amino acids. It is located on chromosome 14q11. It consists of 24 exons and the predicted size of its protein product is 1259 amino acids.

The *RPGRIP1* protein is a structural component of the ciliary axoneme. One of its functions is required for normal disk morphogenesis. It has been shown that *RPGRIP1* is essential for RPGR function and is also required for normal disk morphogenesis.

Recently, in an in vivo investigation of *RPGRIP1* function and its physical interaction, it has been shown that *RPGRIP1* is essential for RPGR function and is also required for normal disk morphogenesis.

Here we report the first observation of the involvement of *RPGRIP1* gene mutations as a cause of CRD in four Pakistani families.

**SUBJECTS AND METHODS**

We studied 20 members of a two generation and 19 members of a three generation consanguineous Pakistani families, 1CRD and 4CRD, respectively. The 1CRD family consists of eight affected and 12 unaffected subjects and the 4CRD family consists of eight affected and 11 unaffected subjects (fig 1A, B). One of the authors (A Aziz) clinically examined all the patients and their unaffected family members. The deterioration in central vision and colour blindness was from an early age in all the patients and there was a rapid loss of vision between the ages of 14 and 16 years. Funduscopy showed a variable degree of fundus granularity and macular degeneration. The affected subject IV.7 (aged 14 years) has a characteristic macular bull’s eye lesion in both her eyes. Full field flash ERG was used to measure functions of both cones and rods. Both photopic and scotopic full field ERG amplitudes were reduced, showing involvement of both photoreceptor systems. However, among the young patients, cone response was reduced more than that of their rod response. Based on family history and clinical diagnosis, the disease was classified as autosomal recessive cone-rod dystrophy (arCRD).

For genetic analysis peripheral blood samples were collected with informed consent from all members of the two families and from 100 ethnically matched control subjects.

**Linkage analysis**

Genomic DNA was extracted from whole blood using the Nucleon II extraction kit (Scotlab Bioscience). DNA was amplified using primers (Research Genetics) for polymorphic microsatellite markers specific for known loci/genes associated with various retinal degenerations according to the conditions described previously.

**Mutation detection**

Exon specific intronic primers were designed from the genomic sequence of the *RPGRIP1* gene (NM 020366). PCR was performed in a 50 µl reaction volume using 1 µg of genomic DNA. The resulting product was allowed to cool slowly to room temperature to maximise the formation of heteroduplexes. Heteroduplex analysis was performed using an automated DHPLC instrumentation (WAVE DNA fragment analysis system, Transgenicom, Crewe, UK). Sample preparation for heteroduplex analysis was carried out by denaturing and renaturing of unpurified PCR products of the carriers (heterozygotes). The temperature conditions required for the successful resolution of heteroduplexes were obtained from the website (http://insertion.stanford.edu/melt.html).

On identification of heteroduplex peaks in the carriers, all family members were sequenced in the forward and reverse directions using a commercially available kit (Big Dye, ABI) and the products were analysed on an ABI Prism 377 automated DNA sequencer. Subsequently, six small families with CRD (5, 6, 7, 8, 9, and 10CRDs) and two with LCA were also included for mutation screening of the *RPGRIP1* gene. To exclude the possibility that the mutations are polymorphisms, 100 ethnically matched control samples were also screened for heteroduplexes. The unpurified PCR products of the control

**Key points**

- *RPGRIP1* gene mutations have previously been reported to cause Leber congenital amaurosis.
- Here we report two novel mutations in the *RPGRIP1* gene that cause cone-rod dystrophy in four Pakistani families.
- A homozygous G to T point mutation was identified in exon 16 of nucleotide 2480 in all the affected members of family 1CRD.
- In the affected members of another CRD family (4CRD), a G to T substitution was found in exon 13 at nucleotide 1639.
- The same G 1639 T mutation was found in two more small families, 5CRD and 10CRD.
samples and the homozygous wild type reference DNA sample were mixed in an equimolar ratio. The mixture was then subjected to a three minute, 95°C denaturing step followed by gradual reannealing from 95-65°C over 30 minutes. The heteroduplex mismatches were detected using the WAVE DNA fragment analysis system.

**RESULTS**

Exclusion studies on both the large families (1CRD and 4CRD) showed linkage with the microsatellite marker D14S1023 (Zmax = 5.17 and 4.21 for the two families respectively at θ = 0.00) at chromosome 14q11, a locus for the RPGRIP1 gene (fig 1A, B). Mutation screening of the candidate gene, RPGRIP1, was carried out to identify the disease associated mutations.

Initially, DHPLC analysis was performed with samples from unaffected carriers of the families. Heteroduplex mismatches were recognised by the appearance of more than one peak in the elution profile. The presence of heteroduplex peaks in the unaffected carriers was convincing enough to do sequencing to identify the exact mutational change.

Sequence analysis of the RPGRIP1 gene for family 1CRD showed a homozygous G to T point mutation in exon 16 in all the affected subjects (fig 2A). This substitution at nucleotide 2480 changes codon 827 from CGC (Arg) to CTC (Leu). This change in exon 16 was not found in any other Pakistani family studied here, the 100 control samples, nor any of the unaffected subjects of family 1CRD.

In the affected members of family 4CRD, a G to T substitution was found in exon 13 at nucleotide 1639 (fig 2B). This point mutation changes codon 547 from GCT (Ala) to TCT (Ser). The same mutation was found in two other small families, 5CRD and 10CRD (fig 2C). No disease associated mutation was observed in the RPGRIP1 gene sequence for the remaining CRD and LCA families examined.

In addition, three polymorphisms were also identified in the RPGRIP1 gene, which include a CTC to CTT (Leu427Leu) polymorphism in exon 16, a G to A sequence change in intron 9 and a deletion (9 base pair) in the intronic region of exon 13, 32 bp downstream from exon 13.

**DISCUSSION**

Homozygous mutations in the RPGRIP1 gene have been reported in a panel of unrelated patients with Leber congenital amaurosis (LCA). In most of these cases, the mutations result in a premature termination codon. To date RPGRIP1 is the only gene that has not been associated with any other retinal disease phenotypes except LCA. LCA represents the severe end of a spectrum of inherited retinal dystrophies while cone-rod dystrophy is a milder condition. It has been suggested that mutations that cause residual RPGRIP1 activity may lead to phenotypes such as RP or CRD which are less severe compared to LCA.

Mapping of two Pakistani families with cone-rod dystrophy to the RPGRIP1 locus supports this hypothesis. The identification of two novel disease associated mutations also indicates allelic heterogeneity of the RPGRIP1 gene. Both novel mutations are in exons encoding domains of RPGRIP1 that are reported to be involved in interaction with the RPGR gene product. Prosite (http://us.expasy.org/tools/scanprosite/) scan predicted the occurrence of an additional, more efficient, casein kinase II phosphorylation site in the Ala547Ser mutated protein, the significance of which is unknown. The second mutation (Arg827Leu) occurs in the major calcium dependent membrane binding module, the CK2 domain of the RPGRIP1 protein. However, the prediction does not indicate any change in the 3D structure of the domain. Functional analysis of this protein would be required to demonstrate the role of these mutations in retinal dystrophies.
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Figure 2  Selected electropherograms of members of the 1CRD and 4CRD families. In each panel, the left electropherogram is for the heterozygous carriers and the right electropherogram is for the patients who are homozygous for the respective mutation. (A) III.4 (carrier, left) and IV.5 (patient, right) from family 1CRD showing a G-T transversion in exon 16 of the RPGRIP1 gene. (B) III.1 (carrier, left) and IV.1 (patient, right) from family 4CRD showing a G-T transversion in exon 13 of RPGRIP1 gene. (C) Two small families, 5CRD and 10CRD, that carried the same G-T mutation in exon 13 of RPGRIP1 gene.
Geographical and ethnic variation of the 677C>T allele of 5,10-methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas worldwide


Since its biochemical characterisation in 1991 and its genetic identification in 1995, the 677C>T allele (T allele) of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene has been a focus of increasing interest from researchers worldwide. The expanding spectrum of common conditions linked with the 677C>T allele now includes certain adverse birth outcomes (including birth defects), pregnancy complications, cancers, adult cardiovascular diseases, and psychiatric disorders. Although several of these associations remain unconfirmed or controversial, their scope is such that it becomes of interest to explore the geographical and ethnic distribution of the allele and associated genotypes. Accurate information on such distribution can contribute to studies of gene-disease associations (by providing reference population data) and population genetics (by highlighting geographical and ethnic variations suggestive of evolutionary pressures), as well as help to evaluate health impact (by allowing estimates of population attributable fraction).

Current population data, however, show gaps and even for some ethnic groups or large geographical areas (for example, China) few data are available. Our aim was to supplement the available data by collecting a large and diverse sample of newborns from different geographical areas and ethnic groups, and to examine international variations in the distribution of the 677C>T allele. We present findings relating to more than 7000 newborns from 16 areas around the world.

**MATERIALS AND METHODS**

The study was conducted under the auspices of the International Clearinghouse for Birth Defect Monitoring Systems (ICBDMS) and was coordinated through its head office, the International Center on Birth Defects (ICBD).

**Sample selection**

Participating programmes, in consultation with the coordinating group, identified a population sampling approach that would be simple yet minimise sampling bias with respect to the MTHFR genotype. We made an explicit attempt to sample systematically the newborn population. Details of each programme’s approach are listed below, and further information is available on request.

Generally, programmes chose one of two approaches. The first approach used regional newborn screening programmes as the source of samples. Typically, such an approach used a

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Our objectives were to characterise the geographical and ethnic distribution of the 677C>T allele (T allele) of the MTHFR gene and its associated genotypes among newborns around the world, using newborn screening programs and birth hospitals. The participants were 7130 newborns of different ethnicities from 16 areas in Europe, Asia, the Americas, the Middle East, and Australia.

• The distribution of the allele showed marked ethnic and geographical variation. The homozygous TT genotype was particularly common in northern China (20%), southern Italy (26%), and Mexico (32%). There was also some evidence for geographical gradients in Europe (north to south increase) and China (north to south decrease). The TT genotype frequency was low among newborns of African ancestry, intermediate among newborns of European origin, and high among newborns of American Hispanic ancestry. Areas at the extremes of the frequency distribution showed deviations from Hardy-Weinberg expectations (Helsinki, Finland, southern Italy, and southern China).

• This study, the largest to date, suggests the presence of selective pressures leading to marked geographical and ethnic variation in the frequency of the 677C>T allele. Geneticists can benefit from these reference data when examining links between the 677C>T allele and health outcomes in diverse populations.

geographically defined birth population. In Atlanta, for example, researchers visited the Georgia newborn screening programme on different days over several weeks and selected a 1 day collection of blood spots received by the laboratory from children whose mothers resided in one of five counties in Atlanta. Discussions with the director of the newborn screening programme indicated that day to day variability in the flow of specimens from birth hospitals to the state laboratory was negligible. The second approach relied on systematic sampling directly from birth hospitals that were part of an established network. In Spain, for example, staff collected specimens from 15 consecutive newborns at each hospital participating in the ECEMC monitoring programme, which includes birth hospitals from across Spain. Details for specific areas are summarised below.

Australia, New South Wales
Specimens were obtained from the New South Wales newborn screening programme, by selecting 100 consecutive newborn screening cards on each of five consecutive days, excluding repeat specimens, for a total of 500 specimens. All maternity units in the state of New South Wales send their specimens to the programme. Specimens consisted of blood remaining after routine newborn screening tests had been completed.

Canada, Alberta
Specimens were taken from consecutive newborns from the provincial newborn screening programme in Alberta. Specimens consisted of the remaining blood spots used by the newborn screening programme. The first 100 specimens of the month were collected each month for four months.

China, northern and southern
Umbilical cord blood samples were collected from newborns from major hospitals in 12 cities in China from March to November 1998. One hundred consecutive samples were requested from each hospital. The hospitals were in cities from southern China (Wuhan, Nanjing, Guangzhou, and Chengdu) and northern China (Yanbian, Urumchi, Changchun, Jinan, Xi’an, Shenyang, Beijing, and Jilin). For homogeneity, only newborns of Han ethnicity were included in the study.

Finland, Helsinki
Specimens originated from newborns in the major maternity hospital in Finland, at the Helsinki University Hospital. Sampling was restricted to babies whose parents were both Finns. Specimens consisted of the remainder of umbilical blood specimens for hypothyroidism screening. The latter are collected for every newborn in Finland.

France, Strasbourg
Specimens were taken from consecutive newborns from newborn screening centres in Département du Bas-Rhin, whose births are also covered by the Strasbourg Birth Defects Registry. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Hungary
Specimens were taken from consecutive newborns. Specimens were collected from the remainder of the blood spots from the two newborn screening centres that operate in Hungary. For twin pairs, only one of the pair, selected at random, was included.

Israel, Tel Aviv
Specimens were taken from consecutive newborns from one major university hospital in Tel Aviv and consisted of blood spots.

Italy, Campania
Specimens were taken from consecutive newborns at three hospitals in Campania (two in Avellino, one in Benevento). Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Italy, Sicily
Specimens were taken from consecutive newborns from the newborn screening programme in south east Sicily. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Italy, Veneto
Specimens were taken from consecutive newborns at one hospital outside the town of Vicenza. The hospital was chosen because it is an area hospital with 1200 births per year that has good obstetric care but does not select for high risk pregnancies. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Mexico
Specimens were randomly selected from blood spots from newborns born in hospitals that are part of the RYCEMC birth defect monitoring network in Mexico. Samples were obtained from the remainder of the blood spot specimens collected for hypothyroidism screening. Selection was stratified to include equal numbers of males and females in the final sample.

The Netherlands, northern region
Specimens were randomly chosen from newborns whose mothers resided in the northern Netherlands. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Spain
Specimens were taken from consecutive newborns in 67 hospitals of the National Health Service throughout Spain. Essentially all babies in Spain are born in such hospitals. These hospitals are part of ECEMC (Spanish Collaborative Study of
Congenital Malformations), which monitors one quarter of all births in Spain. Each hospital contributed specimens for 15 consecutive newborn infants during three selected months. Specimens were collected at the same time as the blood spots for the newborn screening programme.

Russia
Specimens were selected from the neonatal screening programme that collects and banks specimens from 54 maternity hospitals in the Moscow area. All selected babies were apparently free from congenital anomalies.

USA, Atlanta (Georgia)
Specimens were chosen from newborns whose mothers resided in one of the five counties in metropolitan Atlanta, as ascertained from information on the newborn screening card. Staff visited the newborn screening programme four times over two months. At each visit researchers selected at random the specimens collected during one day and collected specimens from the blood spots left over from newborn screening.

Sample determination and data collection
We determined that a sample size of approximately 400 to 500 specimens per area would provide a reasonably precise estimate (plus or minus 3%) for a genotype with 10% frequency. Such a frequency is within the range reported for the homzygous 677C>T (TT) genotype in many European countries and among North Americans of European descent, and is intermediate between the lower frequencies reported in some populations of African descent, and the higher frequencies reported in specimens from Mexico, Italy, and Hispanics in the USA. Although 400 to 500 was the targeted number of specimens per area, smaller sample sizes were accepted, recognising that such samples would provide less precise estimates. For each sample, researchers collected information on sex and race/ethnicity. Ethnicity was determined from the blood spot card (for example, in Atlanta), from maternal interview (for example, Italy, Veneto, Spain), or from the last name or birth place of the parent (for example, Australia). Not all programmes collected all variables.

Human subject protection
Local review boards approved the study. In most cases specimens were anonymised before testing. In all cases, personal identifiers were removed before data were provided to ICBD for epidemiological analysis.

Laboratory testing
Genomic DNA was isolated from blood spots collected on filter paper. The presence of the C>T change within the MTHFR gene creates a HinfI restriction site that can be detected by restriction enzyme digestion followed by electrophoresis. Amplification of the MTHFR gene by the polymerase chain reaction and detection of the T allele was performed using protocols based on the method of Frosst et al.1

Five programmes (USA, China, Israel, Mexico, The Netherlands) tested their own specimens. All other specimens were tested at a single laboratory (Naples, Italy). The laboratories participated in proficiency testing to ensure inter-laboratory agreement.

---

Table 1 Allele and genotype frequency of the 677C>T allele of the MTHFR gene by area and ethnic origin

<table>
<thead>
<tr>
<th>Area</th>
<th>Sample No</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Genotype (%)</th>
<th>Allele frequency</th>
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</table>

*TT, two 677C>T alleles; CT, one 677C>T allele; CC, no 677C>T allele.
consistency. The proficiency testing consisted of the preparation of punches from 12 blood spots in one laboratory (CDC) with a mix of genotypes (CC, CT, and TT). These genotypes were confirmed by sequencing. Punches from these blood spots were then sent to the other laboratories, which performed DNA extraction followed by genotype assay. The laboratories were blinded to the genotype of these specimens as well as to the relative proportion of the genotypes. Results from each laboratory were sent back to CDC for evaluation. With the exception of one sample, which could not be amplified by four of the six laboratories, results showed complete agreement across laboratories.

Statistical analysis
We computed the confidence intervals for proportions using the Wilson score method without continuity correction. Deviation from Hardy-Weinberg equilibrium was tested by chi-square analysis. In addition to allele frequencies, we present genotype frequencies for the homozygous TT genotype (two 677C>T alleles), the heterozygous CT genotype (one 677C>T allele), and the homozygous CC genotype (no 677C>T alleles).

RESULTS
We present data on 7130 newborns from 16 areas in the Americas, Europe, Russia, China, and Australia. Amplification rates for blood spots by geographical source were the following: Italy, Sicily 89%, Italy, Campania 76%, Italy, Veneto 73%, Spain 69%, France, Strasbourg 78%, Finland, Helsinki 95%, Hungary 95%, Russia, Moscow 95%, Australia, New South Wales 79%, Canada, Alberta 77%, USA, Atlanta 96%. By comparison, a large Irish population based study using newborn blood spots successfully genotyped 85% of collected samples.

Prevalence by geographical area and ethnicity
The allele and genotype distribution, by area and ethnicity, is presented in table 1. The prevalence of the homozygous TT genotype (two 677C>T alleles) is visually summarised in fig 1 for those groups with at least 50 subjects tested.

The distribution of the 677C>T allele showed regional and ethnic variations. For example, the prevalence of the homozygous TT genotype was 10-12% in several areas in Europe (for example, Spain, France, and Hungary). However, the prevalence appeared to be lower (4% and 6%, respectively) in Finland, Helsinki and the northern Netherlands, whereas in some areas in southern Europe it was much higher (26% and 20% in Campania and Sicily, respectively). In the Americas, the frequency of the homozygous TT genotype was higher in Mexico (32%), intermediate in Atlanta (11% among whites), and somewhat lower in Alberta (6%). In Australia, TT prevalence was 7.5% among whites.

Genotype varied by ethnicity as well as by geographical location. For example, TT homozygosity was more common among newborns from Mexico or those born in Atlanta of Hispanic origin, intermediate among newborns of European ancestry (for example, in Europe and North America), and lower among newborns of African ancestry (for example, in Atlanta and Veneto, Italy). However, a range of genotype frequencies was evident even within broad ethnic groups. For example, TT homozygosity among whites ranged from as low as 6% in Alberta (Canada), to 7.5% in New South Wales (Australia), to 11% in Atlanta (USA), to the high values already noted for Italy. For other ethnic and racial groups, such estimates are more unstable because of the smaller number of specimens, but it is worth noting an apparently low frequency of TT homozygosity among newborns of Asian origin from Australia and Atlanta.

Hardy-Weinberg equilibrium
The observed distribution of the three genotypes (CC, CT, TT) in most areas was similar to that expected under Hardy-Weinberg equilibrium (table 2). This was true also among males and females separately (data not shown). We found a relative excess of TT homozygotes in Campania, Italy, and an excess of heterozygotes in Helsinki, Finland and southern China (p<0.05).

DISCUSSION
We documented distinctive geographical and racial/ethnic variation in the prevalence of the 677C>T allele of the MTHFR gene among a large international sample of newborns. The several fold variation in the prevalence of the TT homozygous genotype across the study areas (fig 1) was also consistent, in some areas, with the presence of geographical gradients. In Europe, for example, the prevalence of the TT genotype increased in a roughly southerly direction, from low values in the north (4-7% in Finland, Helsinki, northern Netherlands, and Russia), to intermediate values (8-10%) in France and Hungary, to higher values in southern Europe (12-13% in Spain and northern Italy), peaking in southern Italy (20-26% in Campania and Sicily). In North America, the frequency of
TT homozygotes increased from western Canada (Alberta) to south eastern United States (Atlanta) and peaked in Mexico.

Ethnic variation was apparent among and within geographical areas. In metropolitan Atlanta, for example, TT homozygosity was common among newborns of Hispanic origin (15%), intermediate among those of European origin (11%), and low among African-American newborns (3%). These data are consistent with the high prevalence of TT homozygosity among newborns from Mexico in this study and with published data from the population based sample of babies of Mexican ancestry from California. The low prevalence among US blacks is similar to that reported in pooled estimates of five studies on US blacks and three studies from sub-Saharan Africa as well as in later studies from South Africa and Zimbabwe. The intermediate prevalence among whites in Atlanta is consistent with similar rates observed in several European areas in this and several other studies. However, more detailed comparisons are difficult because of the misclassification and imprecision of such ethnic labels.

Of note is the finding in Australia of a lower prevalence of the TT genotype among whites (7.5%) compared to previous reports. Also, we noted a relatively low prevalence of the TT genotype (5.8%) among the random sample of white newborns in Alberta (Canada), compared to the frequency (11%) reported in a previous study from Quebec (Canada). The latter study differed from ours in that newborns were enrolled from a single university hospital in Montreal and were selected, by design, so that their birth weights were at or above the 10th centile.

The high frequency of TT homozygotes observed in this study among newborns from Mexico, northern China, and southern Italy was notable. These findings confirm and extend those previously reported from Mexico and southern Italy. Why such high rates of TT homozygosity occur in these regions is unclear, given the apparently limited ethnic, genetic, or environmental commonalities among such areas. Researchers have suggested the possibility of heterozygote advantage with respect to the risk for neural tube defects. However, such a hypothesis remains unconfirmed. Nevertheless, further exploration of gene-gene and gene-environment interaction might help to identify the evolutionary pressures favouring a high prevalence of this gene variant in certain areas and ethnic groups.

The impact of such geographical and ethnic variation on the distribution of disease in the population is unclear. For example, one would predict high rates of neural tube defects, whose risk appears to be increased nearly two-fold in the presence of 677C>T homozygosity in those geographical areas or ethnic groups with a high frequency of this genotype. The evidence supporting such relations is mixed. For example, the data are consistent for Mexico and northern China, which not only have a very high frequency of the TT genotype but also high rates of neural tube defects. Furthermore, within China, rates of neural tube defect are higher in the north (where the TT homozygous genotype is more common) than in the south. In the United States, the rates of neural tube defects historically have been higher among Hispanics, intermediate among non-Hispanic whites, and lower among African-Americans, a trend that follows the relative frequency of the TT homozygous genotype.

There are, however, notable exceptions. In southern Italy, for example, the TT genotype is common, but the rate of neural tube defects is not particularly high. Nevertheless, such exceptions are not entirely unexpected, because environmental and nutritional factors are likely to modulate considerably the genetic risk for neural tube defects. In fact, these exceptions might prove particularly valuable when investigating the aetiological heterogeneity and the role of interactions in the occurrence of neural tube defects.

Similar analyses are possible with respect to other outcomes. For example, recent meta-analyses showed associations of the TT genotype with ischaemic heart disease, deep
venous thrombosis, and perhaps stroke.24 Like neural tube defects, these health outcomes are subject to interacting risk factors and therefore the relation between genotype and outcome at a group level is likely to be complex. Nevertheless, researchers seeking to understand such relations might find data such as these on the geographical and ethnic variation of the 677C>T allele helpful.

On a population level, the genotype distribution associated with the T allele was generally consistent with Hardy-Weinberg expectations. However, a few significant deviations did occur, mostly at the ends of the frequency spectrum. An excess of TT homozygotes was observed in southern Italy (where the allele was common), whereas the reverse was observed in Finland (Helsinki) where the allele was uncommon. Though these two deviations from Hardy-Weinberg expectations could be the result of chance and multiple statistical testing, they might also suggest the presence of local selective pressures.

In interpreting the findings of this study, one should consider its strengths and limitations. Although we attempted to draw unbiased, systematic samples of newborns from defined populations, sampling strategies varied across areas and one cannot be certain that the efforts were always entirely successful. We provide details on sampling procedures as guidance to readers who wish to use part or all of these data. Dealing with race and ethnicity was also a difficult but inescapable challenge. Classifications based on self report and particularly on the birth place of the parent or last name are unsatisfactory to varying degrees. Thus, we present our data (table 1) either stratified in two groups (the main ethnic group and all other groups combined), or present data only for the major ethnic group (for example, Han Chinese). While this approach does not solve the difficulties entirely, it decreases the misclassification inherent in defining the many smaller ethnic groups that coexist in many areas. Other limitations of this study include the lack of coverage from many areas of the world, including most of Africa, the Middle East, Latin America, and the Indian subcontinent.25

Another challenge of this study was addressing measurement error in genotyping. One might speculate, for example, that deviations in Hardy-Weinberg equilibrium may be the result of genotyping errors. However, inter-laboratory consistency and quality control measures showed remarkable agreement among laboratories. In addition, the same laboratory that assayed samples from areas showing deviations from Hardy-Weinberg equilibrium also assayed the samples from many areas not showing such deviations, suggesting no systematic laboratory error.

A strength of the study was the ability to assemble systematically relatively large samples from newborns using explicit sampling protocols. Measures were also taken to ensure the reliability and comparability of genotypic data across laboratories, including quality control protocols that involved blind retesting of results and exchange of specimens.

Data from studies such as these can serve several purposes. Geneticists could find them useful when evaluating the distribution of genetic variation in human populations and its role in genetic susceptibility to disease. For example, population data might help geneticists reassess controversial associations such as that between MTHFR genotypes and risk for Down syndrome,20–22 for which the evidence favouring the association was largely derived from comparisons with convenient samples of controls. As discussed previously, these genetic data can help to interpret prevalence gradients of disease, such as the well known geographical gradients of neural tube defect occurrence. Similarly, huge amounts of data on other outcomes, such as other birth defects, pregnancy complications, certain cancers, adult cardiovascular disease, and certain psychiatric disorders,23,24 could be called upon to interpret the prevalence gradients noted in this and other studies. Our data are offered as a contribution to such investigation.

Population data on the 677C>T variant might also help population and public health geneticists assess the potential impact of preventive measures based on environmental modifications. For example, some adverse biochemical effects of the thermolabile enzyme coded by the T allele, such as the increase in total plasma homocysteine, appear to be reversible by increasing the consumption of the B vitamin folic acid.26 If the effect of folic acid varies by genotype, then the overall impact in the population of fortification or supplementation programmes might vary predictably once the genotype distribution is known.

Finally, a practical outcome of this collaborative study was to show the feasibility of conducting such genetic surveys using existing networks of hospitals, birth defect registries, and research institutions. Other research groups have similarly selected and examined large and representative samples of newborns from single states or countries (for example, California27 and Ireland28) and generated genotype prevalence data. We tried to expand such efforts to an international scale, and suggest that, with appropriate planning, such international networks can use their access and experience in community based studies to provide core data on the population distribution of common gene variants. These data in turn can serve as the foundation for studies of genetic variation and its role in increasing or decreasing disease risk.

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Small babies receive the cardiovascular protective apolipoprotein ε2 allele less frequently than expected

C Infante-Rivard, E Lévy, G-E Rivard, M Guiguet, J-C Feoli-Fonseca

A newborn whose weight for gestational age and sex is less than expected, based on population standards, is considered as having intrauterine growth restriction (IUGR); a cut off at less than the 10th centile is often used to define IUGR. Causes of IUGR remain unclear although a number of fetal and maternal risk factors have been identified. Increased early morbidity and mortality, as well as, possibly, less than optimal neuropsychological development, have been reported as consequences of IUGR. The association between restricted fetal growth and adult chronic diseases (often referred to as the Barker hypothesis) is now considered robust and possibly causal.

Apolipoprotein E (apoE) is one of the key regulators of plasma lipid levels as it affects hepatic binding, uptake, and catabolism of several classes of lipoproteins. The apolipoprotein E gene (APOE) codes for the apoE protein; in animal models, underexpression of the APOE gene and lack of the apoE protein result in increased susceptibility to atherosclerosis, whereas gene overexpression displays anti-inflammatory, anti-proliferative, and atheroprotective properties. ApoE has also emerged as a central factor in various biological processes such as immunoregulation, control of cell growth and differentiation, and brain development. The three common allelic variants at the APOE locus (ε2, ε3, ε4) code for three major apoE protein isoforms (E2, E3, E4). These isoforms differ from one another only by single amino acid substitutions, yet these changes exhibit functional consequences at both the cellular and molecular levels. In previous studies, children who carry the ε4 allele and those who carry the ε2 allele have been shown to have, respectively, higher and lower total cholesterol and low density lipoprotein (LDL) cholesterol than those with the ε3/ε3 homozygous genotype.

Despite mounting evidence indicating the participation of APOE polymorphisms in various developmental processes involving cell growth and differentiation, atherosclerosis, brain development, and other disorders, we found no report on the relation between APOE polymorphisms and IUGR. Given the data suggesting changes in cardiovascular disease (CVD) risk with the different apoε alleles, and the data showing that growth restricted babies are at higher risk of CVD, we thought it justified to examine if there is or is not preferential transmission of the apoε alleles from parents to IUGR cases.

METHODS

Study subjects

We carried out a study of IUGR in relation to thrombophilic polymorphisms. Cases were newborns whose birth weight was below the 10th centile for gestational age and sex, based on Canadian standards. All cases seen at our centre between May 1998 and June 2000 who were born alive after the 24th week of gestation and without severe congenital anomalies were eligible for the study if the mother agreed to participate. The project was approved by the Institutional Review Board of the hospital. Informed consent was signed by the mother to collect cord and maternal blood. During that period, 505 newborns met the criteria for cases and 493 participated in the study (97.6%). In the original study, we also included controls, which are not used in the present report. Midway through this study, we started to collect buccal swabs from the fathers of babies to be included in the final phase of the study. The goal was to analyse case-parental trios (mother, father, newborn) to test for association and linkage. Among the fathers, contacted, 86% provided genetic material. We genotyped 449 newborns and 440 mother-newborn pairs for APOE (89% of all case pairs); there was enough DNA from the buccal swabs remaining to genotype APOE in 194 fathers (78% of fathers providing DNA). Genotyping of all three family members was complete for 170 trios.

Laboratory investigation

Human genomic DNA was extracted from whole blood samples (mothers and newborns) or from buccal swabs (fathers), as previously described. Briefly, PCR reactions were performed with a final reaction volume of 50 l, using 50-100 ng of DNA template per tube under the following conditions: an initial DNA denaturation step at 94°C for three minutes before adding the mixture containing the Taq DNA polymerase enzyme; this was followed by a 40 cycle sequence of primer annealing at 62°C for 30 seconds, extension at 72°C for one minute, and denaturation at 94°C for 30 seconds with a 10 minute final extension step at 72°C. The PCR APOE primer sequences were as follows: 5′ CGGGACCGGCTGTCCAAAGGA 3′ (forward) and 5′ CGGGCCCGGCCCTGTAQAC 3′ (reverse).

Allele specific oligonucleotide hybridisation assays were performed as described by others. PCR products were denatured, divided into aliquots, and blotted onto nitrocellulose membranes. Positive and negative controls were included on each membrane. Specimens of family members were assigned randomly to membranes. After hybridisation and washing, the membranes were read using PhosphorImager (Molecular

Key points

- A newborn whose weight for gestational age and sex is less than expected, based on population standards, is considered as having intrauterine growth restriction (IUGR).
- The APOE gene has three common allelic variants (ε2, ε3, ε4), which result in functional consequences at both the cellular and molecular levels. The ε2 allele has been associated with lower total cholesterol and low density lipoprotein cholesterol.
- We studied the transmission of the three APOE alleles from heterozygous parents to newborns with IUGR and found a significantly reduced transmission of allele ε2.
- Because the ε2 allele has been associated with a lower risk of cardiovascular disease, while babies born with growth restriction are reported to be at higher risk for such disease later in life, our data seem to reconcile these two observations.
Statistical analysis

The general goal of the analysis was to determine if transmission of an allele from the parents to the newborn departs from the expected probability (50%). A statistically significant departure from the expected (or preferential transmission) is indicative of linkage and association. First, we used the transmission disequilibrium test (TDT) that analyses the transmission of alleles from heterozygous parents to their affected child. To analyse the trait defined as presence of IUGR, we used the Family Based Association Test (FBAT) program. FBAT replaces missing values for the unobserved parental genotype using the distribution of offspring genotype. We also used the TDT program from STATA, which does not replace missing values; both programs use a McNemar test for the expected. Overall, transmission of apo \(\epsilon 2\) significantly departed from the expected probabilities. Removing the 32 trios where probands had signs of placental infarction did not change any of the conclusions: for allele \(\epsilon 2\), 16 transmissions were observed while 27 were expected \((p=0.002)\), whereas for allele \(\epsilon 3\) these were 132 and 199, respectively \((p=0.02)\), and 44 and 44.5, respectively, for allele \(\epsilon 4\) \((p=0.74)\). The global chi-square was 9.78 \((p=0.007)\).

Table 3 shows the results of the analysis with the FBAT program when using the birth weight of the probands as the trait. Although there was a tendency towards a deficit in transmission of allele \(\epsilon 2\), the results were not statistically significant. Removing the 32 trios where probands had signs of placental infarction did not change any of the conclusions (data not shown).

A logistic regression analysis with transmission of allele \(\epsilon 2\) as the outcome of interest confirmed the results of all previous analyses showing a significantly reduced transmission of allele \(\epsilon 2\) (results not shown). Introducing race, sex, birth weight, gestational age, and maternal smoking, each in turn in the model, did not affect the transmission probabilities as measured by the likelihood ratio (LR) chi-square statistic comparing nested models. Removing from this analysis, which uses complete trios, the 10 trios where probands had signs of placental infarction did not materially change the results.

Finally, using the GASSOC program, we rejected the null hypothesis that the genotype relative risks were null based on the LR statistic \((7.7, 2\ df, p=0.02)\). Under the additive model, allele \(\epsilon 2\) was associated with half the risk of allele \(\epsilon 3\), used as the reference allele \((relative\ risk (RR) = 0.44, p=0.008)\), while the risk was close to null for allele \(\epsilon 4\) \((RR=0.82, p=0.39)\). The dominant model was also compatible with the data \((score\ statistic = 6.8, 2\ df, p=0.03)\). The recessive model could not be estimated well with only one case carrying the \(\epsilon 2/\epsilon 2\) genotype.
Removing from the analysis the 10 trios where the proband had signs of placental infarction did not materially change the results.

**DISCUSSION**

Using a family based study design and related statistical tests, we consistently found a significantly reduced transmission of allele ε2 to newborns affected with intrauterine growth restriction; in other words, allele ε2 seems protective against IUUGR. Overall, results indicated significant linkage disequilibrium (linkage and allelic association) between the APOE polymorphisms and IUUGR. On the other hand, using the probands’ birth weight as the trait, we did not observe significant deviation in the number of transmissions from the expected. One possible explanation for the latter results is the relatively limited variation in birth weight among cases. Removing the trios where placental infarction was found on routine pathological examination did not alter any of the conclusions, although the results for the presence of IUUGR were even more statistically significant. This could point to a different cause for the growth restricted newborns with placental infarction because their inclusion in the analysis seems to dilute the effect; however, there was only a small number of such newborns and placental infarction reported on gross examination was not confirmed histologically in this study.

If we assume that allele ε2 is protective against later cardiovascular disease, the fact that it is less often transmitted to babies who are born small gives support to the Barker hypothesis. Indeed, the hypothesis suggests that newborns with small body size are more prone to later cardiovascular diseases. To our knowledge, there are no previous reports showing linkage disequilibrium between the APOE locus and IUUGR.

The relation between the apo ε2 allele and cardiovascular disease is complex. A protective role for the allele in the development of CVD has been reported11 and this seems particularly marked in younger people.12 The apo ε2 allele is also associated with lower LDL cholesterol levels,13 14 15 as well as with a survival advantage16 17 which could be the result of a reduced risk of cardiovascular disease. However, the apo ε2 allele is also associated with higher triglyceride levels and this seems particularly marked in younger people. 

In conclusion, our results are indicative that the apo ε2 allele is transmitted significantly less often than expected among babies whose birth weight for gestational age and sex was below the 10th centile. Because the apo ε2 allele has been associated with a lower risk of cardiovascular disease, and babies born with growth restriction have been found to be at higher risk of cardiovascular disease, our data reconcile these two observations. IUUGR is a complex disease about which we know little in terms of mechanisms. In a previous study, we had excluded the role of thrombophilic polymorphisms as potential contributing causes for IUUGR.18 19 The results of the present study may suggest an underlying atherosclerotic mechanism for IUUGR. Despite the plausibility of our results, they need to be replicated in independent studies.

**ACKNOWLEDGEMENTS**

The study was supported by grants from the Canadian Institutes of Health Research (MA-14705 and MOP-53069) and the Research Foundation of CHUME Sainte-Justine. Claire Infante-Rivard holds a Canada Research Chair (James McGill Professorship).

**REFERENCES**


**Table 3** Results of the FBAT on birth weight among probands with intrauterine growth restriction

<table>
<thead>
<tr>
<th>Allele</th>
<th>S*</th>
<th>E(S)†</th>
<th>Z score‡</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε2</td>
<td>-2.55</td>
<td>3.41</td>
<td>-1.57</td>
<td>0.11</td>
</tr>
<tr>
<td>ε3</td>
<td>17.46</td>
<td>12.24</td>
<td>0.87</td>
<td>0.27</td>
</tr>
<tr>
<td>ε4</td>
<td>9.96</td>
<td>12.91</td>
<td>0.20</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Using a global χ² (2 df) = 2.35, p=0.31.

*Test statistic for the observed number of transmitted alleles.
†Expected value of S under the null hypothesis of no linkage and no association.
‡Each allele is compared to the others.

17 18 19
Pathogenic mutations but not polymorphisms in congenital and childhood onset autosomal recessive deafness disrupt the proteolytic activity of TMRPSS3

Y J Lee, D Park, S Y Kim, W J Park

Congenital hearing loss occurs in approximately 1 in 1000 live births and 60% of these cases are hereditary.1,2 Non-syndromic autosomal recessive deafness accounts for about 70% of congenital hereditary hearing loss cases. To date, at least 33 genetic loci have been identified.3 Mutations in the TMRPSS3 gene, which encodes a transmembrane serine protease, are responsible for non-syndromic autosomal recessive deafness, DFNB8 and 10. Pathogenic mutations were found in the LDLRA (low density lipoprotein receptor class A) and SCAR (scavenger receptor cysteine rich) domains as well as the serine protease domain.3


Key points

- Mutations in the TMRPSS3 gene, which encodes a transmembrane serine protease, are responsible for non-syndromic autosomal recessive deafness, DFNB8 and 10. Pathogenic mutations were found in the LDLRA (low density lipoprotein receptor class A) and SCAR (scavenger receptor cysteine rich) domains as well as the serine protease domain.

- We have assayed the proteolytic activity of the TMRPSS3 proteins containing pathogenic mutations or polymorphisms using a yeast based protease assay.

- All the six tested pathogenic missense mutations disrupted the proteolytic activity of TMRPSS3, while two non-pathogenic polymorphisms did not affect the activity.

- The disruption of proteolytic activity of TMRPSS3 is tightly correlated with the pathogenesis of deafness.
Figure 1. Principle of sGASP. In a yeast strain lacking invertase activity (suc2), a fusion protein is expressed in which invertase is linked to the truncated lumenal domain of an integral Golgi membrane protein, STE13, by a short substrate sequence containing linker. In the absence of cleavage of the substrate sequence, the invertase moiety remains anchored to the Golgi membrane (A). However, upon cleavage of the substrate sequence by a specific protease, invertase is released into the periplasmic space where it degrades sucrose to glucose and fructose. As a result, transformants are able to grow on selective plates containing sucrose as the sole carbon source (B). The structures of the STE13-substrate-invertase fusion protein and STE13-TMPRSS3 are shown in (C). Targeting proteases to Golgi apparently augments the protease-substrate interaction and hence the proteolysis.

these mutations occur not only in the catalytic domain, but also in the LDLRA and SRCR domains involved in interactions with extracellular molecules, the molecular mechanism for pathogenesis is unclear. In this study, we determine whether these missense mutations affect the proteolytic activity of TMPRSS3.

MATERIALS AND METHODS

Generation of wild type and mutant TMPRSS3 constructs

The luminal region of TMPRSS3 (accession number AB038157) was amplified by PCR and fused to the truncated STE13. The PCR primers used in this amplification (5′-AGC CCT CGA GGT TGC TGT GAA GTA C-3′ and 5′-AGG GGC CCG TCA GGT TTT TAG TCT GCT G-3′) was also used for generation of mutated TMPRSS3. Missense mutations and polymorphisms were introduced by PCR based mutagenesis. Primers sets containing the nucleotide alterations are as follows: D103G (5′-CAA AGG GGA GGA GTA CGG CTG TTG GGA G-3′ and 5′-AGG GGC CCG TCA GGT TTT TAG TCT GCT G-3′), C194F (5′-AGG GAG GGA TTT GCC TCT GCC G-3′ and 5′-AGA GGC AAA TTC CTC CTC C-3′), W251C (5′-CAT GAT TAC TAC TCG TCA GCA GCC CAC CCG CAC GAG C-3′ and 5′-AGC TAC TAC AGC GGC G-3′), P404L (5′-CAC AGC GGC GCG TCT CTC TAG GAA CAT G-3′ and 5′-AGC TAC TAC AGC GGC G-3′), C407R (5′-CAC AGC GGC ATT TGG CAC TAC GAG ACA GGC GGC-3′), D173N (5′-AGG GAG GGA TTT GCC TCT GGC-3′), D125V (5′-AGG GAG GGA TTT GCC TCT GGC-3′), D103G, D125V, D173N, W251C, P404L, and C407R; were all defective in proteolytic activity (panels II and III). Pathogenic missense mutations (D103G, R109W, C194F, W251C, P404L, and C407R), were all defective in proteolytic activity (panels IV-IX). In contrast, the two polymorphisms G111S and I253V did not affect the proteolytic activity of TMPRSS3 (panels X and XI). Our data indicate that the pathogenesis is strongly correlated with the defective proteolytic activity of TMPRSS3.

We additionally examined two reported polymorphisms, D173N and A426T, which could not be clearly categorised as non-pathogenic. Our results showed that the D173N mutant possesses full proteolytic activity, while that of A426T is significantly diminished (panels XII and XIII). We suggest that D173N is a non-pathogenic polymorphism, while A426T could be pathogenic in certain circumstances (for example, when the critical substrates of TMPRSS3 become slightly less cleavable owing to gene alterations, the A426T mutation in TMPRSS3 could worsen the situation and eventually lead to pathogenesis). It is interesting to note that D173 is not conserved, while A426 is highly conserved among TMPRSS3.

Recently, the epithelial amiloride sensitive sodium channel (ENaC) was suggested to be a potential substrate of TMPRSS3. It was suggested that TMPRSS3 proteolytically activates ENaC, which might control important signalling pathways in the inner ear. Consistent with this report, our data indicate that disruption of the proteolytic activity of TMPRSS3 is tightly correlated with the pathogenesis of hearing loss. It remains to be seen how the mutations in the LDLRA
and SRaC domains affect the proteolytic activity of TMPRSS3. It may be possible that these domains are necessary for proper folding or assembly of the catalytic domain or protease substrate recognition and binding.

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Hearing impairment is the most prevalent sensory disorder and genetic causes are thought to be responsible for over 60% of the cases in developed countries. Inherited hearing impairment is highly heterogeneous from both the clinical and genetic points of view. It varies in age of onset, severity, and audiological characteristics, and it can be associated or not with other clinical features (syndromic or non-syndromic hearing impairment). Genetic transmission includes autosomal (dominant and recessive), X linked, and maternal inheritance patterns. This unparalleled heterogeneity is well illustrated by the fact that over 70 loci in the nuclear genome have been reported to be involved in non-syndromic hearing impairment, and about 30 genes have been isolated from their critical intervals. Furthermore, a number of different mutations in several genes of the mitochondrial genome are responsible for syndromic and non-syndromic forms of hearing loss.

Mutations responsible for maternally inherited non-syndromic hearing loss are so far confined to only two genes in the mitochondrial genome. These include mutations 7510T>C and 7517T>C in the tRNA<sup>ser</sup>(UCN) gene, and 1095T>C and 1555A>G in the gene for the 12S rRNA. This last mutation is responsible for a dual phenotype, since it also confers increased susceptibility to the ototoxic action of aminoglycoside antibiotics. Most of these mutations have been reported in a small number of families from several countries, with the exception of 1555A>G, which seems to be more frequent than the others, although its real prevalence remains to be determined in most populations. Remarkably, in Spain it accounts for about 15-20% of all familial cases of non-syndromic hearing loss, irrespective of their mode of inheritance and age of onset (our unpublished results). In a majority of these patients, the hearing loss is not attributable to aminoglycoside ototoxicity. A phylogenetic analysis of mitochondrial DNA (mtDNA) haplogroups, performed on 50 unrelated Spanish families, showed that the 1555A>G mutation could be caused by over 30 independent mutational events, occurring in mtDNA haplogroups which are common in all European populations. These data indicate that the high detection rate of this mutation in Spain is not the result of a single major founder event, at least with regard to the mitochondrial genome. Given the high prevalence of the 1555A>G mutation in Spain, and the possibility of preventing aminoglycoside ototoxicity in mutation carriers, its detection has become a priority in routine genetic testing. In contrast to other mutations in the mtDNA, which are frequently heteroplasmic, the 1555A>G mutation has been found in homoplasy in all but one of the families reported so far. In that study, the mutation was found in heteroplasy in three subjects, with the proportion of mutant copies (the “mutation load”) ranging from 85-94%. Here we report the genetic and clinical characterisation of six novel unrelated Spanish families segregating the 1555A>G mutation in heteroplasmy, with a wide range of percentages of mutant copies in a total of 19 subjects.

**Key points**

- Mutation 1555A>G in the 12S rRNA gene of the mitochondrial genome is responsible for non-syndromic hearing loss, as well as for increased susceptibility to the ototoxicity of aminoglycoside antibiotics.
- In almost all the cases reported so far the mutation was found in homoplasy. Here we report the clinical and genetic characterisation of six Spanish families with sensorineural hearing loss, totalling 19 subjects with heteroplasy for 1555A>G.
- The proportion of mutant copies ranged from 3.75-96.60%. Subjects carrying less than 20% of mutant copies were asymptomatic or had a mild hearing loss, whereas heteroplasmic subjects with over 52% of mutant copies suffered from moderate to severe hearing loss.
- Taking the six families together, there is a correlation of the mutation load with the severity of the hearing loss. However, when studying the families separately, this correlation is confirmed in three of them and excluded in another.
- Our study illustrates the difficulties in extracting general principles from the analysis of the genotype-phenotype correlation regarding the 1555A>G mutation.

**METHODS**

**Subjects**

Familial cases of non-syndromic hearing loss were collected with the only criterion of having at least two affected subjects. Our collection procedure did not cause any other bias, such as preferential selection of large pedigrees or compatibility with maternal inheritance. A total of 649 unrelated Spanish families were enrolled in the study. After getting informed consent, peripheral blood samples were obtained from all participating family members, and DNA extraction was performed by standard procedures.

**Mutation detection**

Screening for the 1555A>G mutation was carried out by PCR amplification of a 339 bp DNA fragment containing the mutation site, followed by digestion with restriction endonuclease HaeIII, as described previously. In the wild type allele, digestion results in two fragments of 216 bp and 123 bp. The mutation specifically creates a novel restriction site, and so digestion results in three fragments (216 bp, 93 bp, and 30 bp).

**Quantification of the mutation load**

The proportion of mutant copies was quantified by detection of fluorescently labelled PCR products separated by capillary electrophoresis. A 359 bp DNA fragment was amplified with
RESULTS AND DISCUSSION

At least one subject from each of the 649 collected families was tested for the presence of the mitochondrial 1555A>G mutation, the result being positive in 105 families (16%). In the positive cases, a search for mutation carriers was performed on all the remaining participating relatives. This screening showed heteroplasm for the mutation in 19 subjects from six unrelated families, which also included 12 subjects with the mutation in homoplasy (fig 1).

All of the mutation carriers in the families with heteroplasm were studied clinically. There were records of treatment with aminoglycoside antibiotics in only two subjects (S138 II.2 and S160 II.2, streptomycin). Other environmental factors were excluded as causes of hearing loss in all the subjects except for one syndromic feature, which were found. Conductive hearing loss was ruled out by otoscopic examination, tympanometry with acoustic reflex testing, and use of the tuning fork tests. Pure tone audiometry, testing for air and bone conduction, confirmed that the hearing loss was bilateral and sensorineural in all affected subjects. Audiograms for air conduction are shown in fig 1. There were no vestibular symptoms except in patient S141 I.2, who reported episodes of positional vertigo. Patients S141 I.2, S141 II.3, and S338 II.1 reported bilateral tinnitus.

As expected, the pattern of transmission of the 1555A>G mutation was consistent with maternal inheritance in all the families (fig 1). The proportion of mutant copies was determined in the 19 heteroplasmic subjects and ranged from 3.75-96.60% (fig 1). The mutation load in the offspring of a heteroplasmic mother was highly variable. For instance, subject S338 I.2 (52.14% of mutant copies) has two sons with the mutant allele in homoplasm within our detection limits, and another son with values close to homoplasm for the wild type allele (3.84% of mutant copies). A wide variation is also observed in the offspring of subject S138 I.2 (fig 1). In addition, pedigrees S160 and S068 have some relevant characteristics. Subject S160 I.2, homoplasmic for the mutant allele within our detection limits, has two heteroplasmic daughters (94.74% and 96.15% of mutant copies, respectively). This result indicates that subject S160 I.2 keeps some wild type copies at least in the germine. In peripheral blood, the wild type allele would have been lost or would be in a proportion small enough to go undetected. As regards pedigree S068, heteroplasmic subject II.5 has two sibs (a brother and sister) who are apparently homoplasmic for the wild type allele. However, he also has a son of her sister (III.1) who is affected by bilateral sensorineural hearing loss, more severe in the high frequencies. Two hypotheses may explain these data. First, I.2, the mother of subjects II.1, II.3, and II.5, would have inherited the 1555A>G mutation in heteroplasm. The mutation would remain in II.5, but would have been lost in II.1 and II.3. If this were the case, the hearing loss in subjects II.1 and III.1 would have a cause different from the 1555A>G mutation, which is a plausible explanation given the genetic heterogeneity of non-syndromic hearing loss. It should be taken into account, however, that the characteristics of the hearing loss in subjects II.1 and III.1 closely resemble those of other members of the family, but it is also true that high frequency hearing loss is the most common type. The second hypothesis concerns the possibility that peripheral blood from subjects II.1, II.3, and III.1 contained a very small, undetected, proportion of mutant copies (apparent homoplasm) or none at all (real homoplasm). However, the mutation load in the inner ear would be large enough to be pathogenic in subjects II.1 and III.1. Were this the case, its implications would be relevant for genetic diagnosis (see below).

We investigated the effect of heteroplasm on the severity of the hearing loss. Subjects carrying less than 20% of mutant copies were asymptomatic (S138 II.1 and II.2, S297 III.2, S338 II.3 and II.4), or had a mild hearing loss (subject S297 IV.1, with a U shaped audiogram). Conversely, the remaining 13 subjects, with percentages of mutant copies between 52.14% and 96.60%, suffered from hearing loss. Two of them, monozygotic twins from family S141 (II.1 and II.2) with 70.93% and 64.48% of mutant copies, respectively, had a mild hearing loss for high frequencies. Five others (S068 II.5 and S138 II.3, II.4, II.5, and II.6), with percentages of mutant copies between 69.78% and 96.60%, had normal hearing for low and middle frequencies, but suffered from moderate or severe hearing loss for high frequencies. In the remaining six cases, the audiogram shape was sloping, affecting both middle and high frequencies (S141 II.3, S160 II.1, and S338 II.2; mutant copies ranging from 52.14% to 94.74%) or all the frequencies (S138 II.1, S141 II.1, and S160 II.3; mutant copies ranging from 61.03% to 96.15%). In this last group, subject S138 II.3 had a history of treatment with streptomycin. The study of 10 subjects from families S068, S160, and S338, carrying the mutation in homoplasm, showed that eight of them had hearing losses which were more severe than those of their heteroplasmic relatives (only one homoplasmic subject, S160 II.2, had a history of treatment with streptomycin). The two remaining cases (S160 II.5 and II.6) were asymptomatic, but it should be considered that they are younger than their four affected sibs, and may be below the age of onset.

A statistical analysis of our data for all the six families showed significant correlation of the mutation load with the hearing thresholds, for all the frequencies (125-8000 Hz range) (fig 2A), and for only the high frequencies (2000-8000 Hz range) (fig 2B). However, these results must be interpreted cautiously, as indicated by intrafamilial analysis. In three families (S068, S160, and S338), the severity of the hearing loss clearly correlates with the mutation load. Regarding family S141, the proportion of mutant copies influences the severity, but this seems to be modulated also by age. Conversely, no apparent correlation is observed between the severity of the hearing loss and the mutation load in four sibs from family S138 with mutant copies ranging from 69.78% to 96.60% (II.3-II.6).

In subjects with only high frequency hearing loss, it was difficult to ascertain their age of onset, since frequently they were not aware of their hearing loss. However, a majority of cases in the germline in heteroplasm reported that their hearing loss first manifested in adulthood (between 17 and 50 years of age). In contrast, in eight out of 10 subjects carrying...
The mutation in homoplasmy, onset was in early childhood (between 1 and 5 years of age).

The study of the genotype-phenotype correlation in subjects carrying the 1555A>G mutation in homoplasmy, which are the vast majority of the cases reported so far, has shown considerable heterogeneity in age of onset, evolution, severity, and other audiological features of the hearing loss resulting from this mutation. This variability has been attributed to the influence of both environmental and genetic factors. Undoubtedly, aminoglycoside antibiotics induce a severe worsening of the hearing loss in mutation carriers. In addition, there is in vitro evidence of the influence of the nuclear background.

Figure 1 Pedigrees of the six Spanish families segregating the 1555A>G mutation in heteroplasmy. A question mark inside a symbol is used to represent subjects whose clinical status could not be ascertained. Age (in years) and audiograms are shown below or to the right of subject symbols. Hearing level (in dB) is plotted versus sound frequency (in Hz). Since the hearing loss was sensorineural in all cases, only results for air conduction are depicted. Circles, right ear; crosses, left ear. For each subject, the proportion (%) of mutant copies (mean of three independent experiments (standard deviation)), estimated from DNA from peripheral blood, is indicated below the audiogram.
in modulating the phenotype caused by the 1555A>G mutation.\textsuperscript{24, 25} Also, the hypothesis of the existence of nuclear genes acting as modifiers of mitochondrial hearing loss has recently received strong support.\textsuperscript{26} The existence of a not negligible percentage of cases with heteroplasm (5.7% in our sample of 105 patients with the 1555A>G mutation) adds more complexity to the picture. Our study of a set of 19 heteroplasmic subjects illustrates the difficulties in extracting general principles from the analysis of the genotype-phenotype correlation regarding this mutation. First, among our heteroplasmic cases, most of the subjects carrying less than 20% of mutant copies were asymptomatic, whereas all of the subjects with mutation loads higher than 52% suffered from hearing loss. This suggests that there is a threshold in mutation load for manifestation of clinical symptoms. However, it is also known that there exist subjects homoplasmic for 1555A>G, who are asymptomatic (for example, subjects S160 II.5 and II.6). Second, statistical analysis of our data indicates a significant correlation of the severity of the hearing loss with the mutation load when considering the six families altogether. However, when studying the families separately, this correlation is confirmed in three of them and excluded in another (family S138). This situation may be because of intrafamilial differences in the nuclear background modulating the phenotype, and/or individual variability in mutation load in peripheral blood and inner ear. In fact, it has been reported that the level of heteroplasm for a given mutation can vary among different tissues within the same person.\textsuperscript{26}

The conclusions of our study are relevant for genetic diagnosis of mitochondrial mutations that are responsible for non-syndromic hearing loss. The estimations of mutation load obtained from mitochondrial DNA from peripheral blood may not always reflect accurately the real situation in the inner ear. In extreme cases, the mutation load may be pathogenic in the inner ear and remain undetectable in blood. Therefore, in large families with several affected subjects and a clear maternal inheritance of the disorder, several probands from different branches in the pedigree should be tested before excluding the presence of the mutation. This issue may be critical for prevention of aminoglycoside ototoxicity in subjects whose carrier status would go unnoticed.

Although the last few years have witnessed great advances in the understanding of mitochondrial pathogenesis, there are many important issues that remain unsolved, such as the basis of tissue specificity and the mechanisms by which a heteroplasmic mutation segregates and is fixed. Further investigation of these matters is needed to improve genetic counseling regarding the 1555A>G mutation.

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3 Hereditary Hearing Loss home page (Van Camp G).


Frequent inherited hearing impairment in two unrelated Spanish patients

GJB2 is frequently inherited in double heterozygosity with mutant alleles in white populations. The most frequent mutation (35delG) accounting for up to 86% of the cases is inherited mainly as an autosomal recessive trait. Hearing loss have been reported, and 16 genes have been identified. The gene encoding connexin26 (GJB2) is inherited as an autosomal recessive trait. The most frequent condition is a severe or profound hearing loss of prelingual onset, which is inherited mainly as an autosomal recessive trait. The most frequent condition is a severe or profound hearing loss of prelingual onset, which is inherited mainly as an autosomal recessive trait. The gene encoding connexin26 (GJB2) is inherited mainly as an autosomal recessive trait. The gene encoding connexin26 (GJB2) is inherited mainly as an autosomal recessive trait.

Uniparental disomy of chromosome 13q causing homozygosity for the 35delG mutation in the gene encoding connexin26 (GJB2) results in prelingual hearing impairment in two unrelated Spanish patients

A Álvarez, I del Castillo, A Pera, M Villamar, M A Moreno-Pelayo, T Rivera, J Solanellas, F Moreno

Homozygosity in two unrelated Spanish patients

GJB2 is frequently inherited in double heterozygosity with mutant alleles in white populations. The most frequent mutation (35delG) accounting for up to 86% of the cases is inherited mainly as an autosomal recessive trait. Hearing loss have been reported, and 16 genes have been identified. The gene encoding connexin26 (GJB2) is inherited as an autosomal recessive trait. The most frequent condition is a severe or profound hearing loss of prelingual onset, which is inherited mainly as an autosomal recessive trait. The gene encoding connexin26 (GJB2) is inherited mainly as an autosomal recessive trait. The gene encoding connexin26 (GJB2) is inherited mainly as an autosomal recessive trait.

Inherited hearing impairment is a highly heterogeneous group of disorders with an overall incidence of about 1 in 2000 newborns. In approximately 70% of cases, the auditory impairment is not associated with other clinical features, that is, it is non-syndromic. The most frequent condition is a severe or profound hearing loss of prelingual onset, which is inherited mainly as an autosomal recessive trait. The gene encoding connexin26 (GJB2) is inherited mainly as an autosomal recessive trait.


Key points

- Mutations in the gene encoding the gap junction protein connexin26 (DFNB1) loci on 13q12 are responsible for up to 50% of all cases of autosomal recessive hearing impairment in most populations, the 35delG mutation being the most frequent in white populations.
- Here we report two unrelated cases of homozygotes for 35delG whose biological fathers were not carriers of the mutation. The study of the segregation of polymorphic genetic markers showed uniparental (maternal) disomy of chromosome 13, causing homozygosity for the mutation. In both cases, the disomic maternal gamete may have resulted from non-disjunction of chromosome 13 in meiosis II.
- These two cases represent the first description of UPD(13) with an abnormal phenotype, and they are also the first cases of UPD resulting in non-syndromic hearing impairment.

Here we report another inconsistency in the segregation of markers in the 13q12 region in two unrelated cases of subjects with prelingual hearing impairment. In these two cases, uniparental disomy of chromosome 13 caused homozygosity for
the 35delG mutation in the GJB2 gene, resulting in the hearing defect.

MATERIALS AND METHODS

Informed consent was obtained from all the subjects that were enrolled in this study. Peripheral blood samples were obtained, and DNA extraction was performed by standard procedures.

Screening for the 35delG mutation was carried out by PCR amplification of a 122 bp DNA fragment including the first 34 codons of the GJB2 gene, using the following primers: forward, 5′-CAAACCGCAGCTAGTGAAG-3′; reverse, 5′-GATAATGCAGAAAAATGAAGG-3′. The forward primer was 5′ end labelled with a fluorescent dye (TET, 6-FAM, or HEX, depending on the mutant allele). The PCR reaction was carried out in a total volume of 15 µl including 20-40 ng of genomic DNA from the patient, 10 pmol of each primer, 2.5 nmol of each dNTP, 1.5 mmol/l of MgCl2, and 0.75 units of AmpliTag Gold DNA polymerase (Perkin-Elmer), in the buffer provided by the manufacturer. PCR was performed using standard conditions with an annealing temperature of 59°C. Protruding tails of adenine nucleotides, which are added by the AmpliTag Gold DNA polymerase to the 3′ ends of the DNA product during the PCR, were eliminated by treatment with T4 DNA polymerase (Roche), under the conditions recommended by the manufacturer. Then, samples were resolved by capillary electrophoresis in an Abi Prism 310 Genetic Analyzer (Applied Biosystems). The rationale of this procedure is to detect the loss of one nucleotide in the mutant allele. Then, the presence of the mutation was confirmed by DNA sequencing of a second PCR product.

Primers and PCR conditions for the amplification of the microsatellite markers used in this study have been previously reported.1

RESULTS

During the routine screening of subjects with non-syndromic prelingual hearing impairment for the 35delG mutation, we found two unrelated cases, E112-3 and E232-3, who were homozygous for 35delG, and had a relevant characteristic in common. In both cases, the mother of the patient carried the mutation, but the father did not. Both cases were sporadic, not having any other affected relative. In family E112, there was also a brother with normal hearing who was a carrier of the 35delG mutation (fig 1). Both patients and their participating relatives were genotyped for seven microsatellite markers flanking the GJB2 gene within an approximate 2 Cm interval. These included D13S175, D13S1275, and D13S292,2 and D13S1830, D13S1831, D13S1832, and D13S1835.3 The marker order is indicated in fig 1. Subject E112-3 was homozygous for all these markers except for the most distal (D13S292). In addition, haplotype analysis showed that the subject had not inherited any allele from his father for five of these markers (fig 1). His brother had inherited the 35delG mutation from their mother, and there was no segregation inconsistency in the alleles he had received from his parents, as expected. Subject E232-3 was homozygous for the seven markers, and she did not share any allele with her father for six of them, as shown by haplotype analysis (fig 1). False paternity was investigated by genotyping the patients and their parents for a series of highly polymorphic microsatellite markers in other chromosomes. Ten markers were completely informative, namely D15S220, D15S234, D15S425, D7S2420, D7S2459, D14S288, D15S153, D15S205, D16S404, and D21S1252.4 In both cases E112 and E232, the non-maternal alleles of all these markers in the child fitted those of the alleged father, the residual probability of a false paternity being 10^-10. This suggested that the anomalous inheritance would be confined to chromosome 13.

Therefore we genotyped the patients and their parents for a set of 13 additional markers from the whole long arm of chromosome 13, evenly distributed at intervals of about 10 Cm (fig 1). In patient E112-3, there were two markers with homozygosity for an exclusively maternal allele (D13S217 and D13S265). There was heterozygosity for all the other markers. For six of them, the patient did not share any allele with his father (maternal heterodisomy). For the remaining five markers, although the patient did share at least one allele with his father, his genotypes were also consistent with maternal heterodisomy. As regards patient E232-3, there were four markers with homozygosity for a exclusively maternal allele (D13S156, D13S265, D13S158, and D13S1265). There was also homozygosity for two other markers (D13S173 and D13S285) for which the patient did share an allele with her father, but the genotypes were also consistent with maternal disomy: there was heterozygosity for the remaining seven markers, all the genotypes being consistent with maternal heterodisomy (for two of these markers, the patient did not share any allele with her father).

DISCUSSION

Altogether, our data indicate that the anomalous segregations of the 35delG mutation in the two cases reported here are the result of uniparental disomy (UPD) of chromosome 13. UPD is defined as the inheritance of both homologues of a pair of chromosomes from only one parent.11,12 This includes isodisomy (two copies of the same parental chromosome), heterodisomy (one copy of each homologue from the same parent), or a mixture of both.13,14 In case E112-3, maternal isodisomy seems to be confined to 13q11-q12, with maternal heterodisomy in the rest of the long arm. Conversely, in case E232-3, maternal isodisomy alternates with maternal heterodisomy along 13q. Depending on the affected chromosome and on the resulting homozygosities, UPD can produce no clinical manifestations or a diversity of abnormal phenotypes. In our two cases, maternal UPD of chromosome 13 results in homozygosity for the 35delG mutation, which causes profound, prelingual non-syndromic hearing impairment. Similar cases of uniparental disomy creating homozygosity for autosomal recessively inherited mutations have been reported in over 20 cases in the literature.15-17 No other clinical signs or symptoms were observed in our two patients, who were aged 15 years (E112-3) and 2 years (E232-3) at the time of examination. In the last few years, several cases of either maternal or paternal UPD of chromosome 13 (UPD13) have been reported.14,15 All of these cases were phenotypically normal, indicating that there are no maternally imprinted genes in chromosome 13.18,19 Our data further support this conclusion.

Mechanisms leading to UPD include (1) gamete complementation, when the zygote arises from the union of a disomic gamete and a nullisomic gamete; (2) trisomy rescue, when a trisomic zygote loses one chromosome in an early mitotic division (one third of the cases result in UPD); (3) monosomy rescue, by mitotic duplication of the monosomic chromosome, which leads to isodisomy for the whole chromosome; and (4) postzygotic errors, when in a normal zygote one chromosome is lost and it is replaced by duplication of its homologue (isodisomy for the whole chromosome).11,12 In our two cases of UPD(13), regions of maternal isodisomy and heterodisomy are observed, which excludes monosomy rescue and postzygotic errors. Although we cannot distinguish which of the two remaining mechanisms (gamete complementation or trisomy rescue) led to UPD(13) in the cases reported here, we can conclude that in both cases a disomic maternal gamete was involved. Formation of abnormal gametes is the result of meiotic non-disjunction events. When non-disjunction errors occur in meiosis I, they result in heterozygosity for centromeric genetic markers (primary heterodisomy), whereas if they occur in meiosis II, they result in homozygosity for the
centromeric markers (primary isodisomy). Recombination events can introduce regions of homozygosity in a situation of primary heterodisomy (secondary isodisomy) and, conversely, they can introduce regions of heterozygosity in a situation of primary isodisomy (secondary heterodisomy). In both E112-3 and E232-3, there are regions of primary isodisomy for centromeric markers, whereas secondary heterodisomy is observed in other regions. This suggests that the non-disjunction event leading to the formation of the disomic maternal gamete took place in meiosis II. The formation of abnormal gametes leading to UPD is frequently found in association with chromosomal rearrangements. In fact, all of the published cases of UPD(13) were associated with Robertsonian translocations or isochromosomes. However, the karyotypes of both patients E112-3 and E232-3 were normal, as well as that of subject E232-1 (father of E232-3) (data not shown). No karyotyping data from the other parents could be obtained.

The two cases reported here are the first cases of UPD(13) with an abnormal phenotype, and they also represent the first cases of UPD resulting in non-syndromic hearing impairment. They were found among a total of 115 unrelated affected subjects who were homozygous for the 35delG mutation (1.7%). Given the high incidence of inherited hearing impairment, this frequency should be kept in mind when performing large screenings of patients for recessively inherited mutations, and UPD should be considered a possibility when anomalous segregation patterns are found in routine genetic testing.

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Background and aims: The aim of this study was to investigate the genetic aetiology of intrahepatic cholestasis of pregnancy (ICP) and the impact of known cholestasis genes (BSEP, FIC1, and MDR3) on the development of this disease.

Patients and methods: Sixty nine Finnish ICP patients were prospectively interviewed for a family history of ICP, and clinical features were compared in patients with familial ICP (patients with a positive family history, n=11) and sporadic patients (patients with no known family history of ICP, n=58). For molecular genetic analysis, 16 individuals from two independently ascertained Finnish ICP families were genotyped for the flanking markers for BSEP, FIC1, and MDR3.

Results: The pedigree structures in 16% (11/69) of patients suggested dominant inheritance. Patients with familial ICP had higher serum aminotransferase levels and a higher recurrence risk (92% v 40%). Both segregation of haplotypes and multipoint linkage analysis excluded BSEP, FIC1, and MDR3 genes in the studied pedigrees. Additionally, the MDR3 gene, previously shown to harbour mutations in ICP patients, was negative for mutations when sequenced in four affected individuals from the two families.

Conclusions: These results support the hypothesis that the aetiology of ICP is heterogeneous and that ICP is due to a genetic predisposition in a proportion of patients. The results of molecular genetic analysis further suggest that the previously identified three cholestasis genes are not likely to be implicated in these Finnish ICP families with dominant inheritance.

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Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide


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Genetics of mitochondrial diseases


Genetics of Mitochondrial Diseases is the 47th volume in the widely read series Oxford Monographs on Medical Genetics. Despite its relatively small size, this book provides a comprehensive review of contemporary understanding of the genetic and biochemical basis of human mitochondrial disease. Unlike other books in the series, this volume focuses on the laboratory science, rather than the clinical genetics. As such it provides an excellent introduction to the field for the clinician interested in understanding the mechanisms at the cellular, organellar, or genome level. It also provides a useful reference text for workers in the field, who, quite naturally, only have expertise in one or two of the many areas covered in the book. The editor, Ian Holt, has managed to obtain contributions from prominent figures in most of the key laboratories working in the field but, unlike many multi-author books, there is little overlap between the chapters. The finished product is almost seamless, and it provides a balanced view of contentious topics (including the thorny subject of mitochondrial DNA replication, repair, and recombination) which is dear to the editor’s heart at present).

The book is arranged in five sections. It begins with mitochondrial structure and function, followed by chapters on pathological mutations of mitochondrial DNA and nuclear genes important for mitochondrial disease. The opening chapter on mtDNA replication, repair, and recombination is particularly well written. This is a difficult and complex topic which is discussed with great clarity, and is followed by useful summaries of what is known about mitochondrial DNA expression, biogenesis, and oxidative phosphorylation. The basic science is followed by a section on molecular pathology, and begins with a chapter on clinical aspects of mitochondrial encephalomyopathies. This provides a useful summary for the laboratory scientist, and therefore complements the overall ethos of the book, which is predominantly laboratory based. The chapters on pathological mutations of mitochondrial DNA, and nuclear DNA provide a succinct and useful synthesis of the published data, suggest new ways of thinking about old problems, and highlight areas where our understanding is weak. The section on nuclear genes focuses on primary mitochondrial diseases, and includes a discussion of the recently mapped genes that cause multiple secondary mitochondrial DNA deletions and mitochondrial DNA depletion, followed by mutations in nuclear encoded respiratory chain subunit genes and complex assembly genes. Many nuclear genetic disorders have an indirect effect on mitochondrial oxidative phosphorylation. In this book the discussion is limited to diseases where the link with mitochondrial metabolism has been directly established, including Friedreich’s ataxia and recessive hereditary spastic paraparesis (SPG7).

The fourth section of the book deals with some contentious and notoriously difficult areas, and the book would not be complete without a section on neurodegeneration and ageing. This is split into three chapters, each of which has a different flavour. The first chapter deals with the cellular mechanisms of mitochondrial disease, focusing on calcium homeostasis, oxidative stress, and protein turnover. This sets the scene for the subsequent chapter on neurodegenerative disease, before a discussion of the various hypothetical mechanisms that could explain the accumulation of mutated mtDNA in normal human ageing. This section has the appeal of current clinical practice—but it covers an area that is likely to have impact on medicine in years to come.

The final section combines chapters on model systems, genetic counselling, and prospects for therapy. Work on cellular and animal models has had a major influence over the last few years. This is comprehensively reviewed, helping the reader understand some of the difficulties that have plagued the area, and highlighting where future developments will lead to significant advances in our understanding of human disease. The section on transmission, prenatal diagnosis, and counselling is without doubt the most revealing of the clinically relevant chapters in the book. It highlights the problems faced in the genetics clinic, and points towards a number of potential solutions that will undoubtedly influence clinical practice in the near future. The final chapter on gene therapy provides an overview of the different strategies that have been employed but, unfortunately, most of these are still very much at the laboratory stage.

Mitochondrial medicine is a rapidly advancing field and, despite the inevitable delay between manuscript submission and eventual publication, this book provides a solid foundation with a genuinely contemporary feel, at the same time offering a taste of the future. In some ways, the book seems misplaced in a series on medical genetics. Unlike other titles, it is definitely not a reference for other health professionals who encounter patients and families with chromosome abnormalities.

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Conflicts of interest: none declared

Chromosome abnormalities and genetic counseling, 3rd edn


The third edition of this outstanding text is an essential resource for counselling for families with chromosome abnormalities. This book has been extensively updated since the previous edition, with new sections on gonadal cytogenetic damage from exposure to extrinsic agents and pre-implantation genetic diagnosis and substantial revisions to the chapters on uniparental disomy and prenatal diagnosis. Data on individual chromosome abnormalities have been listed for several types of aberrations including autosomal ring chromosomes, uniparental disomy states, and mosaicism for an autosomal chromosome. This feature makes the text a comprehensive reference that covers all aspects of clinical cytogenetics with the exception of cancer cytogenetics. The illustrations include colour plates and computer enhanced figures that facilitate visualisation of the chromosome abnormalities. The authors achieve all this using a very readable style. This book is particularly strong concerning the mechanistic aspects of cytogenetic rearrangements and also the consequences of parental chromosome abnormalities (for example, autosomal reciprocal translocations) for childbearing. Aspects of counselling for chromosome aberrations are also provided in an interesting and balanced manner. However, there is as yet relatively little information on comparative genomic hybridisation and array comparative genomic hybridisation, two extremely important cytogenetic techniques that are likely to be extensively used in the future. In addition, the discussion on chromosomal and microdeletion syndromes is largely centred on the cytogenetic aspects of these conditions, in keeping with the focus of the book. Still, this book remains a compulsory text for cytogeneticists, geneticists, and genetic counselors in training and is highly suitable as a reference for other health professionals who encounter patients and families with chromosome abnormalities.

Anne M Slavotinek
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CORRECTION

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The sixth author in the paper by Wilcken et al, published in August 2003 (J Med Genet 2003;40:619–25) is spelled incorrectly; M Redlund should be M Renlund. The authors apologise for this error.